Emerging diagnostic strategies in myelodysplastic syndromes

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Emerging diagnostic strategies in myelodysplastic syndromes

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VRIJE UNIVERSITEIT

Emerging diagnostic strategies in myelodysplastic syndromes

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INTRODUCTION

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A.A. VAN DE LOOSDRECHT.

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Hematopoiesis

Hematopoiesis, literally to make blood, is a biological process in which new blood cells are formed. Under normal circumstances hematopoiesis mainly takes place in the bone marrow. Blood cells are produced by proliferation and differentiation of a small subset of pluripotent hematopoietic stem cells (HSCs) that have the ability to replenish themselves by self-renewal.[1] The progeny of HSCs progresses through various intermediate maturational stages that generate multi-potential progenitors and progenitors devoted to one lineage differentiation (Figure 1).[2] Mature blood cells include: erythrocytes, thrombocytes or platelets, myeloid cells, and lymphocytes. Because mature blood cells have a restricted lifespan, HSCs are required throughout life to replace multipotent progenitors and the precursors committed to individual hematopoietic lineages.

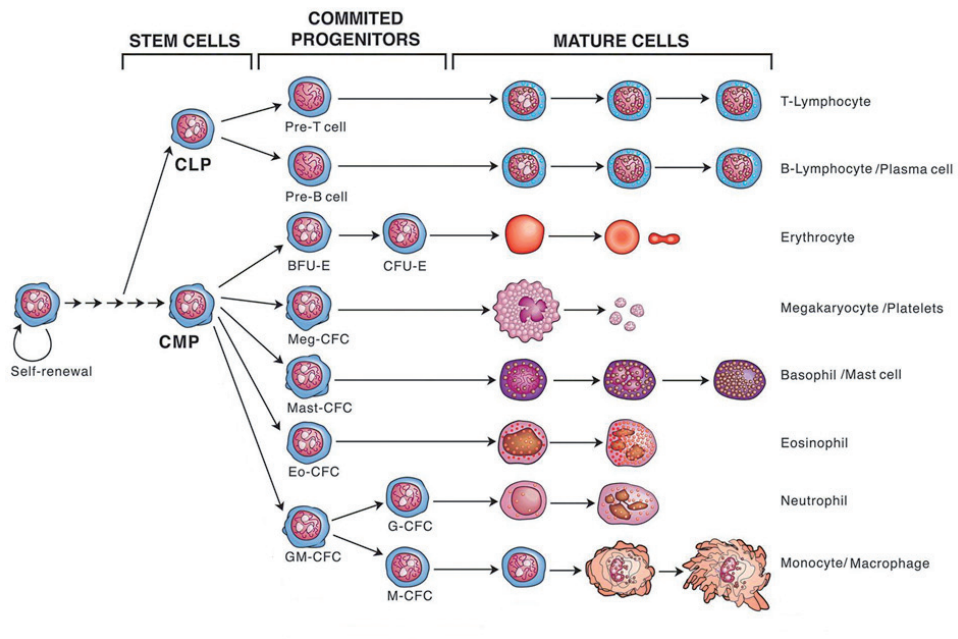

Figure 1. The hematopoietic system where the multipotent HSCs are on the left, and terminally differentiated cells on the right. Abbreviations: CLP common lymphoid progenitor; CMP common myeloid progenitor; BFU-E burst forming unit-erythroid; CFC colony forming cells; CFU colony forming unit. (figure adapted from www.awaremed.com)

Cytopenias

A shortage in (mature) functional cells can occur due to many reasons. Causes can be summarized as: low production (e.g. due to iron deficiency, vitamin B12 and folic acid deficiency, bone marrow failures syndromes), increased use (e.g. due to hemolysis, infections, auto-immune diseases), increased apoptosis (e.g. caused by

medication, splenomegaly, myelodysplastic syndromes), or a combination. Anemia is a shortage of erythrocytes, leukopenia a shortage of leukocytes (neutrophils, monocytes or lymphocytes) and thrombopenia a shortage of thrombocytes or megakaryocytes. Clinical presentations are similar with the type of cytopenia and duration of the disease. A diagnosis should be established based on careful evaluation of the patient history, clinical characteristics and a complete physical examination, followed by analysis of biochemical indicators in the blood that might point to deficiencies or underlying (chronic) illnesses. Once non-clonal causes are considered less likely, the diagnosis myelodysplastic syndromes should be considered.

Myelodysplastic syndromes

Myelodysplastic syndromes (MDS) are a group of clonal bone marrow disorders, characterized by persistent cytopenia(s) and an increased risk of leukemic transformation. The incidence rate of MDS in the Netherlands is about 5.4 cases per 100,000 persons per year in the general population. The incidence rises with older age, with the highest incidence rate at the age of ≥80 years: 84.4 per 100,000 persons a year.[3]

Essential criterion for the diagnosis of MDS is constant cytopenia(s) for more than 3 months; and all other conditions that may cause that cytopenie should have been excluded.[4,5] Landmarks in the diagnosis of MDS are presence of dysplasia in >10% of cells within one or more cell lineages and/or the presence of >15% ring sideroblasts and/or presence of cytogenetic abnormalities typical for MDS and/or presence of a mutation in the splicing factor SF3B1 in combination with >5% ring sideroblasts.[6–8] The cytomorphology, immunohistochemistry and cytogenetic results are used to classify patients in different disease subtypes, as described by the WHO-classification. The WHO-classification is revised every few years. Most of the studies described in this thesis were performed in the "era" of WHO-2008 (**Table 1**).[9] Most recent WHO-classification (2016), removed the terms 'refractory anemia' and 'refractory cytopenia', and replaced them by MDS, with 'unilinear' dysplasia or 'multilinear' dysplasia to provide a more comprehensive description. MDS-RAEB (reactive anemia with excess blasts) is replaces by MDS-EB (**Figure 2**).

Although the WHO 2008 criteria seem quite straightforward, the diagnosis of MDS can still be challenging. Evaluation of dysplasia by cytomorphology is difficult and is not always restricted to a clonal disorder. It might also occur in non-neoplastic disorders, such as vitamin B12, folic acid deficiency or iron deficiency. In addition, inter-observer reproducibility of quantification of dysplasia is poor and experts typically report more frequent and more severe dysplasia than non-experts for the same patient cases.[10]

The diagnosis MDS-unclassifiable (MDS-U) consists of 3 subtypes and is not

Table 1. WHO Classification 2008 for MDS adapted from Vardiman et al.[5]

Classification	Blood findings	Bone marrow findings
Refractory cytopenias with unilineage dysplasia (RCUD): • Refractory anemia (RA) • Refractory neutropenia (RN) • Refractory thrombocytopenia (RT)	Unicytopenia or bicytopenia <1% blasts	Unilineage dysplasia in ≥10% of the cells in one lineage <5% blasts <15% of erythroid precursors are ring sideroblasts
Refractory anemia with ring sideroblasts (RARS)	Anemia <1% blasts	Dysplasia in ≥10% of the cells in erythoid lineage <5% blasts ≥15% of erythroid precursors are ring sideroblasts
Refractory anemia with multilineage dysplasia (RCMD)	Cytopenia(s) <1% blasts No Auer rods <1 x 10^9/L monocytes	Dysplasia in ≥10% of the cells in ≥2 lineage <5% blasts No Auer rods <1 x 10^9/L monocytes
Refractory anemia with excess blasts-1 (RAEB-1)	Cytopenia(s) <5% blasts No Auer rods <1 x 10^9/L monocytes	Uni- or multilineage dysplasia 5-9% blasts Auer rods
Refractory anemia with excess blasts-2 (RAEB-2)	Cytopenia(s) <5-19% blasts Auer rods <1 x 10^9/L monocytes	Uni- or multilineage dysplasia 0-19% blasts Auer rods
MDS, unclassificable (MDS-U)	Cytopenia(s) <1% blasts	Dysplasia in <10% in cells in one or more lineages when accompanied by a cytogenetic abnormality considered as presumptive evidence for a diagnosis of MDS <5% blasts
MDS associated with isolated del(5q)	Anemia Usually normal to elevated platelets <1% blasts	Normal to increased megakaryocytes with hypolobated nuclei <5% blasts No Auer rods Deletion(5q) as sole cytogenetic abnormality

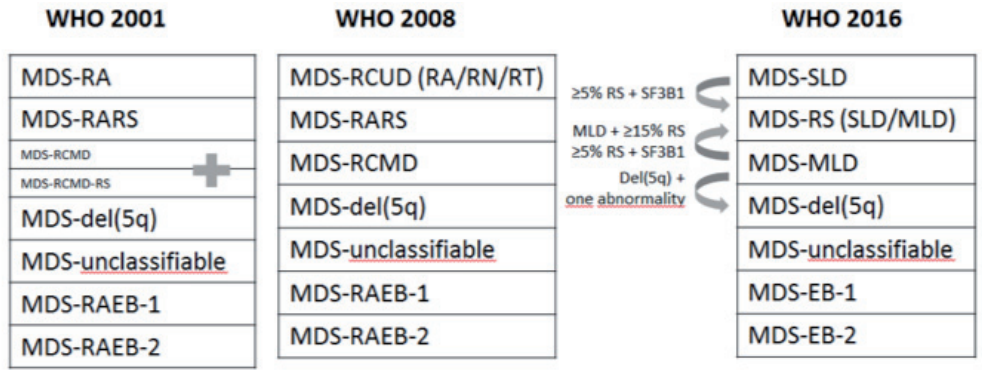

Figure 2. 2001, 2008 and 2016 revisions of the WHO classification for MDS. Abbreviations: RA(RS): refractory anemia (with ring sideroblasts), RCUD: refractory cytopenia with unilineage dysplasia, RT: refractory thrombocytopenia, RN: refractory neutropenia, RCMD(RS): refractory cytopenia with multilineage dysplasia (with ring sideroblasts), del(5): isolated deletion of chromosome 5q, SLD: single lineage dysplasia, MLD: multilineage dysplasia, EB: excess blasts.

necessarily characterized by dysplasia, but by presence of cytogenetic abnormalities or >1% blasts in the peripheral blood without an increase of blasts in the bone marrow. Diagnostic pitfalls by the current diagnostic tools are: 1). 50-70% of the MDS patients do not have any cytogenetic abnormalities, therefore, MDS patients without abnormal karyotype might be unrecognized;[11] 2). Various cytogenetic abnormalities (e.g. a trisomy of chromosome 8) may also be observed in other myeloid neoplasms (e.g. in acute myeloid leukemia or myeloid proliferative diseases);[5] and 3). blast cells in the blood can occur in other hematological malignancies, e.g. in case of marrow fibrosis or population of the bone marrow by other malignant cells. Therefore, additional diagnostic tools are needed to exclude other causes before diagnosing MDS. Molecular biology can be used to suggest other myeloid neoplasms by analyzing presence of frequent genomic aberrations such as FLT3, AML1-ETO, CBF-beta/MYH11, MLL, EVI-1 overexpression, NPM1 mutation, BCR-ABL and Jak2.

Cytopenia of uncertain significance and dysplasia of uncertain significance

Unfortunately, the cause of the cytopenia is not always revealed by this standard diagnostic approach. Patients with an unexplained cytopenia, without a clonal disease, are now categorized as a idiopathic cytopenia of undetermined significance (ICUS).[12,13] Patients with or without cytopenias but with e.g. a clonal mutation are called clonal disease as clonal hematopoiesis of indeterminate potential (CHIP).[14] Patients with clear dysplasia by cytomorphological evaluation, but no cytopenia or other abnormalities in laboratory work-up are classified as idiopathic dysplasia of

undetermined significance (IDUS).[15] Of note, the latter two categories are mostly discovered by coincidence, i.e. during a routine screening.[16]

Introduction to flow cytometry

Overall, more diagnostic tools are needed to distinguish MDS from non-clonal causes of cytopenia and ICUS. Furthermore, with the increased availability of disease-modifying therapies there is a growing need for standardized tools that may predict therapy response.[17] In the recently published European LeukemiaNet guidelines for diagnosis and treatment of MDS, flow cytometry (FC) is added as a recommended tool for diagnostic and prognostic purposes, if performed according to standard European LeukemiaNet guidelines.[18]

Flow cytometry as diagnostic tool in patients with an unexplained cytopenia

By the use of FC physical and histochemical characteristics of hematopoietic cells can be evaluated. MDS-specific FC evaluates the myeloid progenitors, B cell progenitors, maturing myelo- and monocytic, and erythroid cell subsets in bone marrow aspirates. In 2009, the ELN working party on Flow Cytometry in MDS published guidelines concerning recommended methods for cell sampling, handling and processing.[19] In 2012, a paper was published by the same working party that described a minimum consensus panel of antibody combinations to study the myelo-/monocytic and erythroid cell subsets.[20] Knowledge of age-matched normal maturation patterns and expression levels of lineage identifying markers is mandatory to be able to evaluate MDS bone marrow analysis by FC.[21] There is no single specific FC marker for MDS, but the presence of multiple abnormalities predicts the presence of a clonal disorder. Multiple MDS-FC scoring systems describe evaluation of different markers and different scoring strategies.[22–28] An example of a MDS-FC scoring system that applies most of the recommended markers is displayed in **Table 3**.

Most MDS-FC scoring systems focus on two cell compartments: 1). quantifying aberrancies in the myelo-/monocytic cell lineage, analyzing lineage specific marker expression and presence of lineage infidelity marker; 2). analysis of percentage of myeloid progenitor cells and their differentiation-related antigen expression levels and the presence of asynchronous markers and lineage infidelity markers. Scoring systems show a wide range of parameters counted; from few-parametric strategies to complex MDS-FC scoring systems, with a sensitivity and specificity ranging from 69 to 98% and 78 to 93%, respectively.[22–25,27,28,30] The most commonly used diagnostic strategy is the "diagnostic score" according to Ogata et al.[22] This is a simple MDS-FC scoring system based on four parameters: 1). percentage of CD34-positive myeloid progenitors in all nucleated cells; 2). percentage of B cell progenitors within CD34-positive compartment; 3). CD45 expression level of CD34 positive myeloid progenitors (related to CD45 expression level on lymphocytes); 4). sideward light scatter peak channel value of granulocytic cells (related to sideward light scatter peak channel value of lymphocytes). Each abnormality scores 1 point in which scoring ≥2 points is associated with MDS. The sensitivity and specificity of this score are 69% and 89%, respectively. Most of the MDS-FC scoring systems are developed and validated in MDS patient cohorts, comparing groups of MDS patients with patients with non-clonal cytopenia(s) and normal controls. By this approach, FC is able to identify different categories within separate WHO subcategories in MDS.[31,32] This is demonstrated by the detection of multilineage dysplasia in patients where standard cytomorphology only observed unilineage dysplasia.

Until recently, the evaluation of the erythroid cell lineage by FC was challenging due to lack of validation of markers. Nowadays, CD45, CD71, CD235a, CD36, CD117 and CD105 are applied to study the erythroid cell lineage.[25,33,34] An erythroid score (RED score) that evaluates specific erythroid FC markers and hemoglobin level, in addition to the diagnostic score (described above), revealed that the sensitivity of this score is increased to 88%, whereby the specificity remained around 90%.[35] FC analysis of megakaryocytes faces technical challenges due to their scarcity. Efforts are undertaken to overcome these issues by analyzing thrombocytes.[26] Validation studies are ongoing within the ELN working party on Flow Cytometry in MDS.

To summarize, FC has a high sensitivity and specificity in the detection of myelo-/monocytic and erythroid dysplasia. FC should be interpreted not as a sole diagnostic tool in MDS, but can underline the diagnosis in indifferent cases. FC results need to be included in an integrated diagnostic report, together with cytomorphological, cytogenetic and/or molecular findings as dysplastic changes by FC can also be observed in other disorders than MDS.[36] According to the WHO classification, MDS can only be diagnosed when all other causes for the cytopenia/dysplasia are excluded.[9]

Prognostic value of flow cytometry

The International Prognostic Scoring System (IPSS) and the WHO adjusted Prognostic Scoring System (WPSS) and their revised versions are the most applied prognostic scoring systems in MDS. These scoring systems use WHO classification, cytogenetic risk categories, transfusion dependency, bone marrow blast counts, and peripheral blood counts such as hemoglobin level, thrombocyte count and absolute neutrophil count (**Table 4**).[6,7,37,38] Originally MDS-FC models were designed as a prognostic tool that was able to identify different risk categories

Table 3 Example of a MDS-FC scoring system, applying most of the recommended markers. Adapted from Wells et al,[23] scores adjusted as by Cutler et al.[29]

Points	Myeloid progenitors	Granulocytes*	Monocytes*
0	No abnormalities	No abnormalities	No abnormalities
1	<u>≤5% myeloid progenitors with one of the following:</u> Lymphoid markers present (CD2, CD5, CD7, CD19, CD25, CD56) AND/OR Two of the following abnormalities: Decrease in CD45 expression Abnormal expression of CD34 Abnormal expression of CD117 Abnormal expression of CD13 Abnormal expression of CD33 Abnormal expression of HLA-DR Expression of CD11b Expression of CD15	<u>One of the following:</u> Decreased SSC Abnormal CD11b/CD13 Abnormal CD16/CD13 Expression of HLA-DR Abnormal[$] expression of CD33 Asynchronous shift to the left Abnormal expression of CD15 Lack of CD10	<u>One of the following:</u> Abnormal CD45/SSC Decreased/increased number as compared to lymphocytes Abnormal CD11b Abnormal HLA-DR Abnormal CD11b/HLA-DR Abnormal expression of CD14 Abnormal expression of CD13 Abnormal expression of CD33
2	5-10% myeloid progenitors	2-3 abnormalities as described above Presence of CD34 on mature myelo-/monocytic cells Presence of lymphoid markers (such as CD56)	
3	11-20% myeloid progenitors	4 or more abnormalities or one or more abnormalities plus presence of CD34 or lymphoid marker present	
4	21-30% myeloid progenitors	2 or more abnormalities in granulocytic AND 2 or more in monocytic compartment.	
1 Extra	<5% abnormal progenitors without additional granulocyte or monocyte abnormalities	Myeloid/Lymphoid ratio < 1	

*The sum of the granulocyte score and monocyte score has a maximum of 4 points, excluding the described extra point. [$]Decreased CD33 in all cell subsets is seen as a polymorphism, and in that case not scored as abnormal.

within IPSS and IPSS-R risk categories. [39–41] The MDS-FC scores within validated risk categories are heterogeneous. This means, within specific IPSS and IPSS-R risk categories, that FC is able to identify different risk categories based on quantity of FC aberrancies. The presence of multiple abnormalities by FC revealed a poorer prognostic risk within (low risk) MDS based on the IPSS(-R).[42] Moreover, the presence of aberrant myeloid progenitors by FC in patients with <5% blasts by cytomorphology negatively impacts prognosis.[43] Finally, in patients with unilineage dysplasia (RCUD) based on cytomorphology FC may identify multilineage dysplasia which is associated with worse prognosis.[44]

Table 4A The Revised International Prognostic Scoring System (IPSS-R) for MDS

Variable	0	0.5	1	1.5	2	3	4
Cytogenetics	Very good	-	Good	-	intermediate	Poor	Very poor
BM blasts, %	≤2%	-	>2% - <5%	-	>5% - <10%	>10%	-
Hemoglobin	≥10	-	8 - <10	<8	-	-	-
Platelets	≥100	50 - <100	<50	-	-	-	-
ANC	≥0.8	<0.8	-	-	-	-	-

***ANC: absolute neutrophil count**

Table 4B IPSS-R risk categories with corresponding overall survival and risk on leukemic transformation.

Risk category	Score	Median OS (years)	AML 25% (years)
Very low	≤1.5	8.8	NR
Low	>1.5-3	5.3	10.8
Intermediate	>3-4.5	3.0	3.2
High	>4.5-6	1.6	1.4
Very high	>6	0.8	0.73

Treatment modalities in myelodysplastic syndromes

About 80% of the MDS patients will present with an anemia, of which 40% will be treated with supportive care. This means erythropoietin (ESAs) and granulocyte-colony stimulating factor (G-CSF) with or without red blood cell transfusions.[45,46] Other treatment modalities are immune-suppressive strategies such as ciclosporine in hypoplastic MDS or lenalidomide in MDS patients with an deletion

of chromosome 5q. Patients with high risk disease will be treated with conventional high dose chemotherapy with or without an allogeneic stem cell transplantation, or high risk patients are treated with hypomethylating agents (in some cases followed by an allogeneic stem cell transplantation).

Prediction of response to treatment

With the increased availability of disease-modifying therapies in MDS there is a growing need for tools that can predict treatment response and can guide treatment decisions.[17] One of the first FC studies addressing this topic evaluated the presence of aberrant myeloid progenitor cells in predicting response to erythropoiesis stimulating agents. It was illustrated that presence of aberrant myeloid progenitors by FC acted as a significant biomarker for treatment failure.[47] Patients with abnormal progenitor cells by FC showed no response or only short response duration to erythropoietin (EPO) with or without granulocyte-colony stimulating factor. Moreover, in high risk MDS patients receiving hypomethylating therapy (azacitidine), FC proved to have additional value in the prediction of treatment response and FC results correlated with survival.[48] Patients with treatment response after 3 cycles revealed a significant decrease in number of FC abnormalities in comparison to patients without therapy response. The presence of aberrant myeloid progenitors at start of therapy, irrespective of progenitor cell percentages, was significantly correlated with lack of response. In addition, a low number of aberrancies at the start of therapy was correlated with a significantly increased overall survival. Studies evaluating the role of FC in predicting treatment response, e.g. upon immune-modulating agents, are ongoing.

One of the studies that evaluate the use of immune-modulating agents is the *HOVON 89 trial*. This study is a Phase II randomized multicenter study to assess the efficacy of lenalidomide with or without EPO and granulocyte-colony stimulating factor in patients with low and intermediate-1 risk myelodysplastic syndrome (according to the IPSS). The trial is registered at www.trialregister.nl as NTR1825; EudraCT nr.: 2008-002195-10. Patients entered in this study protocol, were either refractory to EPO; loss of response on EPO or were not likely to respond on EPO based on the Nordic predictive response model.

Three add-on studies are attached to this study to verify the efficacy of multiple diagnostic tools in MDS: 1). validation of flow cytometric parameters in myelodysplastic syndromes. 2). identification of genetic aberrations in MDS using high resolution single nucleotide polymorphism (SNP) arrays.[49] 3). Implementation of next generation sequencing in diagnostic and prognostic strategies in MDS. Part of the subjects discussed in this thesis are related to these add-on studies.

Aim of the thesis – part 1

The aim of this thesis is to investigate the efficacy of multiple diagnostic tools in the diagnosis of MDS differentiating clonal from non-clonal causes of cytopenias, focusing on the value of multiparameter FC. This includes the development and validation of erythroid lineage evaluation by FC and addition of selected markers to currently applied MDS-FC algorithms. In addition, investigations were performed to correlate of FC with state-of-the-art molecular techniques performed within the context of a prospective clinical trial within HOVON, (HOVON89).

Chapters 2 and 3 focus on currently used MDS-FC approaches. Chapter 2 aims to investigate the predictive value of current applied MDS-FC scoring systems in detection of MDS, particularly, the role of FC, when other diagnostic tools are inconclusive. Chapter 3 focusses on the FC markers that demonstrate the granulocytic cell differentiation, which forms the cornerstone of currently applied diagnostic strategies for myelodysplastic syndromes (MDS).

Chapters 4 and 5 investigate the role of FC in evaluating the erythroid lineage. Chapter 4 describes an international multicenter study that aims to identify MDS-specific erythroid aberrancies. In Chapter 5 the MDS-specific erythroid aberrancies are/were validated and added to current applied MDS-FC models.

Chapter 6 demonstrates the application of FC and emerging molecular technology in a well-defined low and intermediate-1 risk MDS group within a prospective clinical trial (HOVON89).

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CHAPTER 2

MULTIPARAMETER FLOW CYTOMETRY IS INSTRUMENTAL TO DISTINGUISH MYELODYSPLASTIC SYNDROMES FROM NON-NEOPLASTIC CYTOPENIAS

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Abstract

Mandatory for the diagnosis of myelodysplastic syndromes (MDS) is presence of dysplasia in >10% of cells within one or more cell lineages or presence of >15% ring sideroblasts or presence of MDS associated cytogenetic (CG) abnormalities. Discrimination between neo-plastic and non-neoplastic causes of cytopenias can be challenging when dysplastic features by cytomorphology (CM) are minimal and CG abnormalities are absent or non-discriminating from other myeloid neoplastic disorders. This study evaluated a standard diagnostic approach in 379 patients with unexplained cytopenias and highlights the additional value of flow cytometry (FC) in patients with indeterminate CM and CG. CM reached no diagnosis in 44% of the patients. Here, CG was able to identify two additional patients with MDS; other CG results did not reveal abnormalities or were non-specific. Based on FC results patients without a diagnosis by CM and CG were categorized 'no MDS-related features' (65%), 'limited number of MDS-related changes' (24%), and 'consistent with MDS' (11%). Patients were followed over time in attempt to establish or confirm a diagnosis (median follow-up: 391 days; range 20-1764). The specificity (true negative) of MDS-FC analysis, calculated after follow up was 95%. FC can aid as a valuable tool to exclude MDS when CM and additional CG are not conclusive in patients with cytopenia.

Introduction

Myelodysplastic syndromes (MDS) constitute a heterogeneous group of hematopoietic stem cell disorders, characterized by ineffective hematopoiesis resulting in cytopenias and a variable risk on transformation to acute myeloid leukemia.[1] For the diagnosis of MDS, cytomorphological (CM) assessment of peripheral blood and bone marrow (BM) in combination with cytogenetic (CG) analysis are mandatory.[2–4] The WHO classification is based on CM and CG findings in blood and BM evaluating cytopenias, dysplasia, presence of ring sideroblasts, enumeration of blasts, Auer Rods and cytogenetic findings.[3,4] The minimal CM criterion for MDS is the presence of dysplasia in >10% of the cells in at least one cell lineage, i.e. erythroid, myeloid or megakaryocytic cell lineage. This is challenging, since dysplasia can also occur in myeloid neoplasms other than MDS, or in reactive conditions. Therefore, other possible causes of dysplasia such as deficiencies, or viral infections should be excluded before the diagnosis of MDS can be established.[5,6] A supplementary hallmark in the diagnosis is presence of MDS associated CG abnormalities, present in approximately 40-50% of the patients.[7,8] CG abnormalities described as typical MDS are commonly seen in other myeloid neoplasms.[9,10] For example, a trisomy of chromosome 8 does not lead to the diagnosis of MDS, as this mutation is also seen in myeloid proliferative disorders. Therefore, cytogenetic results should always be interpreted in the context of CM results. In cases of minimal abnormalities by CM and absence of (MDS associated) CG abnormalities there is a need for additional diagnostic tools. Flow cytometry (FC) has proven additional value in the identification of myelodysplasia in large study cohorts of suspected MDS patients.[11,12] The aim of the present study is to evaluate the use of FC as diagnostic tool when CM and CG are not informative in a cohort of patients with unexplained cytopenias.

Patients and methods

Patients

Bone marrow samples of 379 adult patients with cytopenias, defined according to WHO-criteria, sent to our department from January 2009-January 2014 were evaluated. Median age: 66 years (range 20-94), 245 male and 134 female (**Table 1**). In accordance with diagnostic guidelines, CM assessment by at least one experienced hematologist-cytologist was performed.[11,13] The hematologist-cytologist determined the necessity of CG, and molecular biology (MB). FC was performed standardly (supplementary files). Patients were followed over time until a diagnosis was established or verified. The study was approved by the local ethical committee and in accordance with the declaration of Helsinki.

Table 1 Demographics and clinical characteristics.

Cytomorphology	Total	MDS	Other myeloid	Non-myeloid	No diagnosis
	379	101	54	57	167
MDS (N = 101; 27%)					
RCUD	*5*	*5*	-	-	-
RARS	*9*	*9*	-	-	-
RCMD	*56*	*56*	-	-	-
RAEB-1	*10*	*120*	-	-	-
RAEB-2	*13*	*13*	-	-	-
Hypoplastic MDS	*5*	*5*	-	-	-
MDS/MPD	*1*	*1*	-	-	-
Del(5q)	*2*	*2*	-	-	-
Other explanation (N=110; 29%)					
Chronic myelomonocytic leukemia	33	-	33	-	-
Iron incorporation disorder/deficiency	26	-	-	26	-
Myeloproliferative disease	14	-	14	-	-
Aplastic anemia/pure red cell aplasia	12	-	-	12	-
Lymphoma/solid tumor in bone marrow	8	-	-	8	-
Acute myeloid leukemia	7	-	7	-	-
Auto-immune related	5	-	-	5	-
B-Chronic Lymphatic Leukemia	2	-	-	2	-
Vitamin B12 deficiency	2	-	-	2	-
Hairy cell leukemia	1	-	-	1	-
Abnormal plasma cells	-	-	-	1	-
Normal bone marrow (N=18; 5%)					
<10% Dysplasia	101	-	-	-	101
>1 diagnosis possible	17	-	-	-	17
Dry tap/not enough cells	31	-	-	-	31
Median age in years	66	68	73	62	65
range	20-94	28-94	27-85	20-88	22-91
Gender (m/f)	245/134	72/29	43/11	34/23	96/71
Median follow up in days	377	385	333	496	366
range	6-1764	16-1714	9-983	13-1764	6-1716
Karyotype	N=379	N=101	N=54	N=57	N=167
Typical MDS	31 (10%)	21 (23%)	6 (12%)	1 (3%)	3 (2%)
Not typical MDS	45 (15%)	20 (21%)	6 (12%)	4 (10%)	15 (12%)
Normal	229 (75%)	52 (56%)	37 (76%)	33 (87%)	107 (86%)
No results available	*74*	*8*	*5*	*19*	*42*
Flow Cytometry	N=365	N=101	N=52	N=51	N=161
Diagnostic score* 0	98 (27%)	12 (12%)	4 (8%)	12 (23%)	70 (44%)
Diagnostic score* 1	120 (33%)	22 (22%)	8 (15%)	30 (59%)	59 (37%)
Diagnostic score* 2	77 (21%)	34 (33%)	16 (31%)	8 (16%)	20 (12%)
Diagnostic score* 3	52 (14%)	23 (23%)	18 (35%)	1 (2%)	10 (6%)
Diagnostic score* 4	18 (5%)	10 (10%)	6 (11%)	-	2 (1%)
FCSS* 0-1	136 (37%)	18 (18%)	7 (13%)	27 (53%)	84 (52%)
FCSS* 2-3	140 (38%)	41 (41%)	19 (37%)	20 (39%)	61 (38%)
FCSS* 4 or more	89 (25%)	42 (41%)	26 (50%)	4 (8%)	16 (10%)
iFS* no MDS-related features	158 (43%)	15 (15%)	5 (10%)	33 (65%)	105 (65%)
iFS* limited number of changes	84 (23%)	24 (24%)	6 (11%)	15 (29%)	38 (24%)
iFS* consistent with MDS	123 (34%)	62 (61%)	41 (79%)	3 (6%)	18 (11%)

*Evaluation of dysmyelo-/dysmonopoiesis by flow cytometry, the text provides an explanation per score. Abbreviations: FCSS: flow cytometric scoring system; iFS: integrated flow cytometric score.

Flow Cytometry

Data generated by FC were used to calculate validated MDS-FC scores, the diagnostic score and the (modified) flow cytometric scoring system (FCSS), respectively.[14–16] Both scores were integrated into one FC result, as previously described by our group.[17]

Diagnostic score. This four-parameter diagnostic score comprises the percentage of CD34$^+$ myeloid progenitors in nucleated cells; percentage of B cell progenitors within CD34$^+$-compartment; CD45 expression level of CD34$^+$ myeloid progenitors; SSC peak channel value of granulocytes. Each abnormality (compared to reference ranges) scores one point; ≥2 points allocates 'MDS'.[14]

Flow cytometric scoring system. The FCSS evaluates differences from normal regarding percentages, expression levels, maturation patterns and aberrant expression levels of lineage specific and lineage infidelity markers of immature myeloid progenitor cells and maturing granulocytes and monocytes. Patients were categorized: 'no to mild dysplasia' (0-1 points), 'moderate dysplasia' (2-3 points), and 'severe dysplasia' (≥4 points). Cutler et al. modified this score by adding an extra point in case of <5% abnormal progenitors without additional granulocyte or monocyte abnormalities.[15,16]

Integrated flow cytometric score. The iFS combines the diagnostic score and the modified FCSS into one FC result (**Table 2**). The diagnostic score separates patients into two categories: '<2 points' versus '≥2 points'. Second, is the evaluation of the myeloid progenitor cells (SSC low-intermediate/CD34$^+$ and/or CD117$^+$) separating patients into two categories: normal or aberrant myeloid progenitors. Third, is the evaluation of neutrophils and monocytes according to parameters described in ELN guidelines, separating patients into: normal or aberrant myelopoiesis.[18] According to this strategy patients were classified as 'no MDS-related features', 'limited number of MDS-related changes', or 'consistent with MDS'.

Table 2 The integrated flow cytometric score.

Diagnostic flow score	<2	<2	<2	<2		≥2	≥2	≥2	≥2
Aberrant myeloid progenitors	-	-	+	+		-	-	+	+
Aberrant neutrophils (SSC or two or more other aberrancies) **Aberrant monocytes** (CD56 or two or more other aberrancies	-	+	-	+		-	+	-	+
Conclusion**	A	A/B*	A/B*	C		A/B*	B/C*	B/C*	C

The integrated flow cytometric score (iFS) is based on the diagnostic score and the flow cytometric scoring system (FCSS).[14–16] This model is adapted from Van de Loosdrecht et al.[17] Abbrevations FC: flow cytometry; SSC: sideward light scatter; *Choice for "A or B" and "B or C" depends on the kind and number of aberrancies that are encountered; ** A: 'results show no MDS-related features', B: 'limited number of MDS-related changes', and C: 'results are consistent with MDS'.

Analytical strategy
Diagnostic tools were evaluated at two time-points; first after the integration of all results after initial assessment, and second after addition of follow-up data. To be able to evaluate the additional value of FC in the diagnostic strategy in patients with cytopenias, patients without a diagnosis at the second time-point were excluded from the analysis. If patients could were diagnosed with a myeloid neoplasm other than MDS, patients were excluded from further analysis.[19]

Results

Results of cytomorphology and immunohistochemistry at initial assessment
CM categorized 27% patients as 'MDS', 14% patients as 'other myeloid disease', 15% patients as 'non-myeloid disease', and 44% patients as 'non-clonal'. The 'non-clonal' category consisted of 60% patients with '<10% dysplasia in one or more cell lineages', 10% patients with '>1 diagnosis possible' (i.e. dysplasia but also iron incorporation disorder or deficiency), and 19% patients with 'dry tap/not enough cells', and 11% patients with apparently 'normal bone marrow' **(Table 1)**.

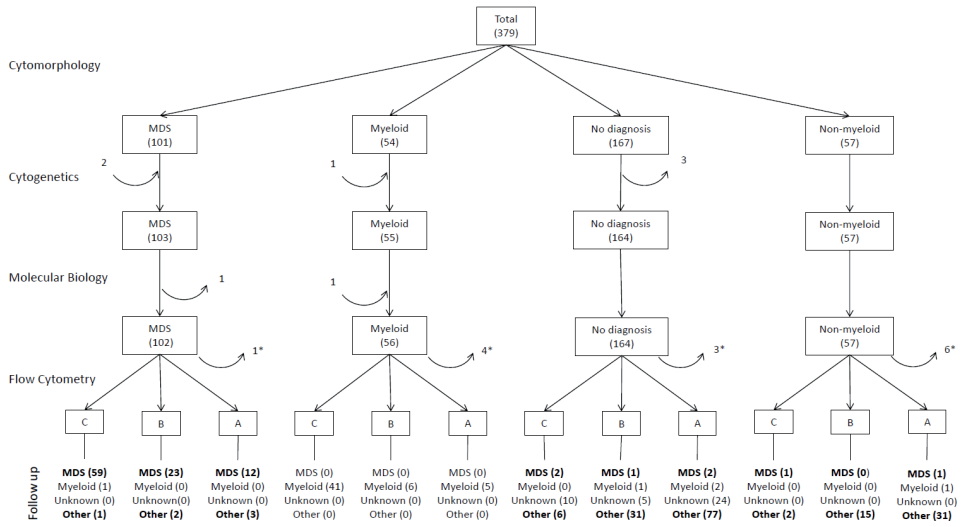
Additive value of karyotyping and fluorescence in situ hybridization
CG analysis was performed in 80% patients. In addition, iFISH was performed in 4% patients. Thirty-one out of 10% patients were labeled: 'typical MDS', 15% were labeled 'not typical MDS' and 75% were labeled 'normal'. CG results within the different categories based on CM are displayed in **Table 1**.
Within the 'non-clonal' category based on CM, CG analysis was performed in 75% patients. Note, for BM aspirates considered normal by CM, CG analysis was not performed. Analysis was 'typical MDS' 2% patients: two patients with a monosomy 7, and one patient with a complex karyotype. The latter and one of the two patients with a monosomy 7 were classified as MDS unclassifiable. The second monosomy 7 patient had a history of chronic myeloid leukemia (CML). However, BCR-ABL was negative at time of analysis; this patient was not classified as MDS.[20] Cytogenetic analysis was 'not typical MDS' in 12% patients. Isolated deletion of chromosome Y was the most frequent abnormality (n=9); none of these results added to diagnosis or exclusion of a specific disease. CG analysis was 'normal' in 86% patients, and thereby not contributory. So, within the no diagnosis CM subgroup, CG was able to identify two additional MDS cases.

Additive value of molecular biology
Molecular analysis was indicated in 29% patients. In 73% patients no abnormalities were reported. Twenty patients had a *JAK2* mutation. *BCR-ABL* was positive in one

patient who was therefore diagnosed as CML. In two patients MB analysis failed due to poor sample quality **(Table 1)**. Within the 'non-clonal' category based on CM, MB was performed in 11% patients. In all of these patients MB revealed no abnormalities.

Flow cytometric results per cytomorphological category
Multiparameter FC analysis focusing on myeloid dysplasia was performed successfully in 365/379 (96%) patients and failed in 4% cases due to poor sample quality. According to the iFS 43% patients FC showed 'no MDS-related features', 23% were labeled 'limited number of MDS-related changes', and 34% 'consistent with MDS'. **Table 1** illustrates the results of the three applied MDS-FC scoring systems within the different CM categories. The iFS is discussed in more detail below.
Flow cytometric results within 'non-clonal' category based on CM. Here, 65% were labeled as 'no MDS-related features', 24% as 'limited number of MDS-related changes', and 11% as 'consistent with MDS', based on the iFS. FC cannot be used as an additional diagnostic tool without knowledge about the predictive value of MDS-FC in patients with a clear diagnosis. Therefore, we first compared FC results in cases where CM could establish a diagnosis.
Flow cytometric results within the 'MDS' category based on CM. Within the 'MDS' category based on CM, 15% of patients were labeled as 'no MDS-related features', 25% as 'limited number of MDS-related changes', and 60% as 'consistent with MDS' (iFS). FC detected dysmyelo-/dysmonopoiesis in 86% MDS patients identified by CM (**Table 1**). Fifteen percent of patients with MDS by CM were not identified by FC. These concerned four patients with isolated dyserythropoiesis; six patients with dysmegakaryopoiesis and dyserythropoiesis; one patient with dysmegakaryopoiesis, dyserythropoiesis, and <10% granulocytic dysplasia; three patients with trilineage dysplasia; and one patient with isolated dysgranulopoiesis. These 15 patients had normal myeloid progenitor cell counts.
Flow cytometric results within 'other myeloid disease' category based on CM. Myeloid neoplasms may show dysmyelo-/dysmonopoiesis by CM. Therefore, it is expected that current MDS-FC scoring systems might label these entities as MDS. Hence, 79% patients within the 'other myeloid disease' category by CM were labeled 'consistent with MDS' by FC; 12% were labeled 'limited number of MDS-related changes'; and 10% were labeled 'no MDS related features' **(Figure 1)**.
Flow cytometric results within 'non-myeloid disease' group based on CM. Within this category 65% were labeled as results show 'no MDS-related features' (true negative), 29% were labeled 'limited number of MDS-related changes', and 6% were labeled 'consistent with MDS' (false positive). The three false positive patients were two patients with reactive BM, and one patient with infiltration of a solid tumor. Interestingly, CM described mild dysmyelo-/dysmonopoiesis in these patients as well.

Figure 1 The flow chart of the study.
The 379 patients entered in de study, subdivided in categories by cytomorphology (CM). The next steps show the addition of cytogenetic (CG) analysis and molecular biology (MB), to the diagnosis by CM. CG alters the diagnosis of three patients into two MDS patients (MDS unclassifiable) and one patient with a myeloid disease (AML). MB alters the diagnosis of one MDS patient into myeloproliferative disease. Initial diagnosis based on CM, CG and MB are subdivided by flow cytometric (FC) results ; A 'FC results show no MDS-related features'; B 'limited number of MDS-related changes'; and C 'FC results are consistent with MDS'.
The bottom of the graph shows the diagnosis after a median follow-up of 377 days (20-1764; N=234). Diagnosis in bold are used to calculate sensitivity, specificity, negative and positive predictive value per diagnostic tool. *Flow Cytometry was not be performed, due to poor sample quality.



Summary of the results after initial diagnostic work-up
After initial work-up a diagnosis could be established in 215/379 (57%) cytopenic patients. CM was able to diagnose 101 MDS patients, 54 patients with 'other myeloid disease', and 57 patients with 'non-myeloid disease'. CG analysis identified two more MDS patients where CM was inconclusive. MB altered the diagnosis of one MDS patient into MPD. After this initial work up: CM, CG and MB revealed no cause for the cytopenia in 43% patients **(Figure 1)**. FC was able to categorize 98% patients, yet the additional value of this tool in the diagnostic work-up can only be established after follow-up.

Confirmation of the results from initial diagnostic work-up during follow-up
Follow-up data were collected to confirm results, in case of lacking explanation for the cytopenia patients were followed until a diagnosis could be established. Follow-up data were present in 282/379 patients with a median follow-up time

of 377 days (range 6-1764 days). Within the 'non-clonal' category by CM follow-up was available in 77% patients, median follow-up time of 366 days (range 6-1716). Patients without follow-up were *excluded* from further analysis. Note, in 7 patients the initial diagnosis of MDS was altered during follow-up, and 7 other patients were diagnosed with MDS within approximately 1 year. After follow-up, 5 patients within the 'non-clonal' category were diagnosed with 'MDS', three were diagnosed with an 'other myeloid disease', 114 were diagnosed with a 'non-myeloid disease', and for 39 there was still no diagnosis **(Figure 1)**.

Specificity and sensitivity of cytomorphology and flow cytometry
As previously described CM is the gold standard in the diagnosis of MDS. Specificity and sensitivity calculated after follow-up were 96% (95% CI: 86.1-99.4), and 94% (95% CI: 87.4-97.8), respectively.
To calculate results for FC at initial work-up, FC results 'consistent with MDS' was scored as MDS; 'limited number of MDS-related changes' and 'no MDS-related features' were scored as no MDS.
Calculation of specificity for FC revealed similar results as for CM: 95% (95% CI: 90.1-97.5). Sensitivity was 61% (95% CI: 51.2-70.9). This lower sensitivity is most likely to be explained by false negative results in MDS patients with only dysery-/dysmegakaryopoiese, not evaluated by current MDS-FC scoring systems.

The role of flow cytometry at initial assessment in patients with cytopenias of indeterminate origin
In 95% 'non-clonal' patients with non-myeloid disease after follow-up, MDS was considered unlikely according to FC results at initial work up. The 6 MDS-FC positive patients were diagnosed as reactive BM, iron deficiency, and medication induced cytopenia. FC predicted MDS in 2/5 cases that developed MDS during follow-up. The three MDS patients not-predicted by FC had dysmegakaryopoiesis with or without dyserythropoiesis by CM. As described earlier, analysis of these lineages is not incorporated in current MDS-FC scoring systems.
Besides, in 7/102 patients within the 'MDS' category by CM at initial assessment this diagnosis was withdrawn during follow-up. In five of these patients FC already predicted MDS to be less likely. From the other two patients with MDS-like FC, diagnoses were altered in MPD and auto-immune associated cytopenia.

Discussion

Myelodysplastic syndromes (MDS) should be considered in every patient with an unexplained cytopenia. The gold standard in the diagnosis of MDS is presence of specific cytomorphological (CM) features and/or presence of MDS associated cytogenetic (CG) abnormalities.[4,7] However, the classification of patients with persistent cytopenias can be challenging when dysplastic features by CM are minimal, and CG is normal or non-specific. As FC is now recommended as diagnostic tool in the diagnostic work-up in MDS it raised the question whether FC was contributory when CM and CG are indeterminate.[11]

In the cohort of 379 patients described here, CM and CG revealed a diagnosis in 57% patients. In the other cases CM in combination with CG found no cause for the cytopenias; this declined to 10% after follow up. In the initially CM-indeterminate patients FC identified 89% of cases as non-MDS. After follow-up, 97% of patients within the latter category did not develop MDS. This illustrates a low probability of development of MDS in patients with normal or minimal changes by FC at initial assessment, in cases where CM was indeterminate and CG analysis was normal. This forfends patients from receiving several bone marrow biopsies, which is not only less invasive for the patient but also saves time and money.

The specificity and sensitivity for FC calculated after follow-up were 95% and 61%, respectively. The found specificity was comparable to the specificity of CM assessment and also comparable to the results of other FC study groups (mean specificity of 94%).[14,18,21–23] Within the studied patient cohort, FC remained highly specific in excluding MDS. The sensitivity of FC in this study was lower than reported by others (average 75%). [14,18,21–23] In general, FC results should always be interpreted in the context of other diagnostic results. The FC-false negative MDS patients showed mostly dysmegakaryopoiesis with or without dyserythropoiesis, not evaluated by current MDS-FC. Other study groups showed that addition of erythroid markers to the FC analysis increased the sensitivity to identify MDS.[21,24,25] A prospective validation study is to evaluate the incorporation of FC erythroid assessments is ongoing.

No diagnosis could be established for the cytopenia in 43% of the investigated patients at initial work-up. According to the WHO 2008 criteria, presence of typical CG abnormalities is diagnostic for MDS. Cytogenetic abnormalities were expected to be present in 40-50% of the MDS cases. Loss of chromosome Y as single abnormality was most commonly seen. However, interpretation of a loss of chromosome Y, here considered not typical MDS, is challenging as it might also be age-related.[10] In the current study none of the patients with loss of Y developed MDS during follow-up.[26]

One could question if 'MDS' patients by CM when based on minimal criteria, without abnormalities by CG, and FC are indeed myeloid clonal diseases or better categorized as idiopathic cytopenia or dysplasia of uncertain significance.[27,28] However, longer follow-up is necessary. Additional molecular analysis, i.e. whole exome sequencing or targeted sequencing of described mutations associated with MDS might contribute in near future.[29]

In conclusion, in this patient cohort of 379 patients with cytopenias, FC showed to be highly instrumental to exclude MDS at the initial diagnostic work-up in cases where CM was indeterminate.

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Supplemantary data

Technical procedures

Cytomorphology and immunohistochemistry

Analysis of blast count and dysplasia was performed after May-Grünwald-Giemsa staining, in a 500-cell differential of nucleated cells in BM smears and in 200-cell differential peripheral blood smears. An iron staining was used for the evaluation of (ring) sideroblasts. In 176/379 (46%) patients BM biopsies were available for immunohistochemistry. Immunohistochemistry results were added to the CM results, in which CM results were decisive. In cases of a dry tap, poor quality of the aspirate, localization of lymphoma, or solid tumor in the biopsy, immunohistochemistry results contributed to CM results. Patients were classified according to WHO 2008 criteria.

CM was used as gold standard to categorize patients 'MDS', 'other myeloid disease', 'non-myeloid disease', or 'no diagnosis' **(Table 1)**. The latter category included patients with '<10% dysplasia', '>1 diagnosis possible', or 'dry tap/not enough cells evaluable'. Patients with cytopenias but apparently normal BM by CM were also labeled as 'no diagnosis'.

Karyotyping and fluorescence in situ hybridization

After CM assessment, CG analysis was considered mandatory in 305/379 patients (80%). A minimum of 20 metaphases was evaluated and described according to the International System for Human Cytogenetic Nomenclature recommendations.[1] Interphase fluorescence in situ hybridization (iFISH) was performed according to standard procedures when no metaphases were present in the cell cultures (11/305) or in some cases to confirm abnormalities found by conventional karyotyping (6/305). MDS-iFISH comprised deletion of chromosome Y, monosomy 5 and 7, trisomy 8 and specific cytogenetic alterations such as del(5q), del(7q), del(17p) and del(20q).

CG results were used to categorize patients as: abnormal i.e. 'typical MDS' or 'not typical MDS', or 'normal', according to the WHO criteria.[2,3] With the exception of deletion of chromosome Y as single abnormality which was labeled 'not typical MDS' as it can be age-related.(4) Patients without 'MDS' by CM who had 'typical MDS' CG results, were diagnosed as MDS according to diagnostic guidelines.[5]

Molecular biology

Molecular biology was performed to rule out other myeloid neoplasms, i.e. a myeloproliferative disease (n = 111; **Table 1**). Molecular assessment was

performed using a panel comprised of the most frequent genomic aberrations associated with myeloid neoplasms such as AML1-ETO, CBF-beta/MYH11, MLL, EVI-1 overexpression, NPM1 mutation, BCR-ABL and Jak2.

Flow Cytometry

Flow cytometry was performed within 24 hours according to ELN guidelines for FC in MDS.(6,7) After ammonium chloride-based erythrocyte lysis and sequentially washing of the samples in phosphate-buffered saline/human serum albumin 0.1%, cells were pre-incubated with human serum immunoglobulins 5 mg/mL for 10 minutes. aThe applied antibody panel outlined in **Table S1**, illustrates that CD45 or CD45/CD34 was used as back-bone marker in a 4-color analysis (2009-2012) and CD34, CD117, HLA-DR and CD45 were used as back-bone markers in the 8-color analysis (2012-2014).(6–8) Antibodies CD2 (FITC), CD5 (FITC), CD10 (PE), CD13 (FITC) CD16 (FITC), CD25 (PE), CD64 (PE), and CD117 (APC), were purchased from DakoCytomation, Glostrup, Denmark. CD7 (PE and APC), CD10 (APC-H7), CD11b (PE and APC), CD14 (APC and APC-H7), CD15 (FITC), CD19 (PE and APC-H7), CD33 (PE and APC), CD34 (FITC, PerCP-Cy5.5, and APC), CD45 (PerCP), CD56 (PE), CD117 (PE), and anti-HLA-DR (APC and V450) from BD Biosciences San Jose, CA, USA. CD36 (FITC) from Sanquin, Amsterdam, The Netherlands. CD45 (KO), CD117 (PC7), and CD123 (PE) were from Beckman Coulter, Miami, FL, USA. IREM2/CD300e (APC) was purchased from Immunostep Diagnostics, Salamanca, Spain.

At least 100,000 white blood cell events (CD45 positive cells) were acquired, including a minimum of 250 CD34 positive cells using a FACSCalibur™ (with red and blue solid-state lasers from BD Biosciences; 2009-2012) or FACSCanto™ II (with red, blue and violet solid-state lasers from BD Biosciences; 2012-2014). Aberrancies with regard to cell percentages (i.e. CD34 myeloid progenitors, B cell progenitors, etc.), sideward light scatter (SSC), expression levels of lineage specific markers and lineage infidelity markers on (im)mature myelomonocytic cells, were evaluated using Cell QuestPro (BD Biosciences) or Infinicyt software (Cytognos) for 4- and 8-color FC, respectively. Gating strategies were performed as previously described by the Dutch Working Party on FC in MDS and the European LeukemiaNet Working Party on FC in MDS.[6,9]

Analytical strategy

The data were analyzed using descriptive and explorative methods. Diagnostic categories were reported using absolute and relative frequencies **(Table 1)**. The data was analyzed using SPSS, Version 20.0, LA.

Table S1A Four color panel of monoclonal antibodies used for immunophenotypic analysis of myeloid dysplasia.

	FITC	PE	PerCP	APC
1			CD45	
2	CD16	CD13	CD45	CD11b
3	CD34	CD11b	CD45	HLA-DR
4	CD36	CD33	CD45	CD14
5	CD36	CD64	CD45	CD14
6	CD15	CD10	CD45	CD34
7	CD34	CD117	CD45	CD13 /CD33
8			CD45	CD34
9	CD5	CD19	CD45	CD34
10	CD2	CD56	CD45	CD34
11	CD13	CD7	CD45	CD34
12	CD13	CD25	CD45	CD34

Table S1B Eight color panel of monoclonal antibodies used for immunophenotypic analysis of myeloid dysplasia.

	FITC	PE	PerCP-Cy5.5	PC7	APC	APC-H7	V450	KO
1			CD34	CD117			HLA-DR	CD45
2	CD16	CD13	CD34	CD117	CD11b	CD10	HLA-DR	CD45
3	CD2	CD64	CD34	CD117	IREM2	CD14	HLA-DR	CD45
4	CD36	*	CD34	CD117	CD33	*	HLA-DR	CD45
5	CD5	CD56	CD34	CD117	CD7	CD19	HLA-DR	CD45
6	CD15	CD25	CD34	CD117	*	*	HLA-DR	CD45
7	CD7	*	CD34	CD117	CD13	*	HLA-DR	CD45

CD45 or CD45 in combination with CD34 was used as back-bone marker in the 4 color analysis (A) and CD34, CD117, HLA-DR, CD45 were used as backbone markers in the 8 color analysis (B). Abbreviations FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCP: Peridinin chlorophyll; PerCP Cy5.5: Peridinin chlorophyll cyanine 5.5; PC7: phycoerythrin-cyanine 7; APC: allophycocyanin; APC-H7: allophycocyanin-Hilite7; V450: 405-nm violet; KO: Krome Orange.

CHAPTER 3

EVALUATION OF NEUTROPHIL MATURATION BY FLOW CYTOMETRY IN MYELODYSPLASTIC SYNDROMES

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Abstract

Due to the wide availability of flow cytometric (FC) markers that demonstrate the granulocytic cell differentiation, this compartment became the cornerstone of currently applied flow cytometric diagnostic strategies for myelodysplastic syndromes (MDS). In this study, we evaluated the diagnostic utility of granulocytic FC markers in a cohort of 239 samples. Bone marrow samples of 123 MDS patients, 72 pathological controls and 24 healthy controls were retrospectively analyzed. Identified FC markers were analyzed in a separate MDS cohort of 70 patients.
The evaluation of all markers in the currently applied MDS-FC neutrophil analysis showed that percentage of the most immature neutrophil population (based on phenotype), premature expression of CD11b, delayed and decreased expression of CD16, and ≥10% of CD56-positive neutrophils were highly suggestive for MDS. Decreased sideward light scatter is frequently observed in MDS, but is at the same frequency affected in pathological controls. The total (decreased) percentage of granulocytes, might underline the diagnosis of MDS but need to be interpreted with caution as they face technical challenges due to hemodilution.
In conclusion, this study identified highly MDS specific neutrophil FC parameters that can contribute to more specific, more sensitive and most importantly more concise MDS-FC panels.

Introduction

Flow cytometry (FC) is a supplementary diagnostic instrument in myelodysplastic syndromes (MDS).[1] With FC the myeloid, monocytic and erythroid cell compartments can be evaluated.[2,3] Due to the wide availability of validated markers that demonstrate the granulocytic differentiation, this compartment became the cornerstone of applied MDS-FC diagnostic strategies.[4–6] Nowadays, more extensive multicolor FC and more advanced software tools have led to a gain in knowledge of normal and dysplastic granulopoiesis.
There is a wide range from few-parametric to complex MDS-FC scoring systems, with a sensitivity and specificity ranging from 69 to 98% and 78 to 93%, respectively.[7] Although many papers are published that describe the granulocytic maturation, there is no study that describes the validity of these markers in particular. In theory, a combination of a few highly specific markers will lead to highly specific identification of MDS. An increased number of markers will increase sensitivity, but will probably result in a less specific model. In this study, we analyzed applied granulocytic FC-parameters in order to identify those markers that are most informative. This data may enable development of more concise MDS-FC panels which are less time consuming and less expensive.

Patients and Methods

We retrospectively analyzed 219 bone marrow samples from 123 untreated MDS patients, 72 non-clonal pathological controls and 24 healthy controls (age-matched volunteers), collected between October 2012 and January 2016. Diagnoses are listed in **Table 1**. The bone marrow samples from the healthy controls served as a references group. FC-parameters that were significantly different between all groups and especially those markers that were significantly different between the MDS cases and pathological controls were validated in a different cohort of 70 MDS patients (**Table 1**).

Samples were processed according to guidelines recommended by the European LeukemiaNet Working Group on FC in MDS (IMDS-flow); data was obtained by 8-colors FC (FACSCanto II; BD Biosciences, San Jose, CA) and analyzed with Infinicyt software (Cytognos, Salamanca, Spain).[7,8] After assigning myeloid progenitors and monocytes (based on SSC/CD45/CD34/HLA-DR), granulocytes were selected by means of SSC/CD45. After elimination of eosinophils and apoptosis the studied neutrophil FC-parameters were: sideward light scatter (SSC; median and mode within different granulocytic subpopulations), median fluorescence intensity (MFI) and/or percentage of CD10, CD11b, CD13, CD15, CD16, CD33, CD45, CD64, HLA-DR,

expression of lineage infidelity markers CD2, CD5, CD7, CD14, CD56 and CD71.[2,9] Seven maturation stages, from most immature to most mature (G1-G7) were determined (population definitions are provided in the supplemental files: **Figures S1-S2**).

Statistical analyses were performed using PASW Statistics version 20.0 (SPSS, Chicago, IL). Reference p-value for significance was <0.05. Differences between patient groups were identified by the Kruskall Wallis test. The Mann-Whitney U test was applied as post-hoc test to analyze inter-group correlations.

Table 1 Diagnoses per patient group

Group	Diagnosis	N
MDS (WHO 2001) (N=123)	RA	2
	RARS	9
	RCMD	42
	RCMD-RS	26
	Isolated del(5q)	1
	RAEB-1	21
	RAEB-2	19
	Hypoplastic MDS	3
Pathological controls (N=72)	Iron incorporation disorder / deficiency	59
	AITP	4
	Vitamin B12 / folic acid deficiency	2
	Chronic illnesses	4
	Medication / cytotoxic	3
MDS validation cohort (N=70)	RA	3
	RARS	2
	RCMD	22
	RCMD-RS	19
	Isolated del(5q)	1
	RAEB-1	9
	RAEB-2	12
	Hypoplastic MDS	2

Abbreviations: RA: refractory anemia; RARS: refractory anemie with ring sideroblasts; RCMD: refractory cytopenia with multilineage dysplasia; RCMD-RS: refractory cytopenia with multilineage dysplasia with ring sideroblasts; Isolated del(5q): an isolated deletion of chromosome 5q; RAEB-1: refractory anemia with excess blasts type 1; RAEB-2: refractory anemia with excess blasts type 2;

Results

Percentage of neutrophils

Peripheral blood neutropenia is often observed in MDS. Therefore, a decreased percentage of neutrophils in the bone marrow may be considered as sign of possible MDS. As described determination of absolute cell counts is challenging by FC.[10] The relative proportion of lymphocytes to myeloid cells can be used to detect abnormal low portions of myeloid cells. Our data revealed that the neutrophil-lymphoid ratio was significantly decreased in MDS as compared to both control groups (**Table 2**). The neutrophil to lymphocytes ratio was not significantly different between patients with <5% or >5% myeloid progenitors (P=0.247). Patients with <5% myeloid progenitors had a median ratio of 5.2; range 0.3-25.8. Patients with >5% myeloid progenitors a median ratio of 3.0; range 0.3-44.5.

Side scatter

Hypogranulation of neutrophils is a cytomorphological characteristic often seen in MDS.[11] The SSC is the FC equivalent of granulation. Within our cohort the SSC (ratio to lymphocyte SSC) proved to be specific for MDS as there was a significant decrease in SSC between MDS and both control groups.

Table 2 Comparison of potential granulocytic aberrancies in MDS, pathological controls and healthy controls

Parameter	Cell subset	MDS/HC	MDS/PC	HC/PC	Total
SSC mode	Total population	<0.001	0.043	0.001	<0.001
	CD10 negative population	ns	ns	ns	ns
	CD10 positive population	<0.001	0.005	0.027	<0.001
SSC median	Total population	<0.001	0.030	<0.001	<0.001
	CD10 negative population	0.001	ns	0.004	0.002
	CD10 positive population	<0.001	0.048	<0.001	<0.001
CD10	Percentage CD10 positive	ns	ns	ns	ns
	MFI	ns	<0.001	ns	<0.001
CD15	MFI	<0.001	ns	<0.001	<0.001
CD45	MFI	ns	ns	ns	ns
CD64*	Percentage	ns	ns	ns	ns
HLA-DR	MFI	ns	ns	ns	ns
Presence of lymphoid markers	CD2	ns	ns	ns	ns

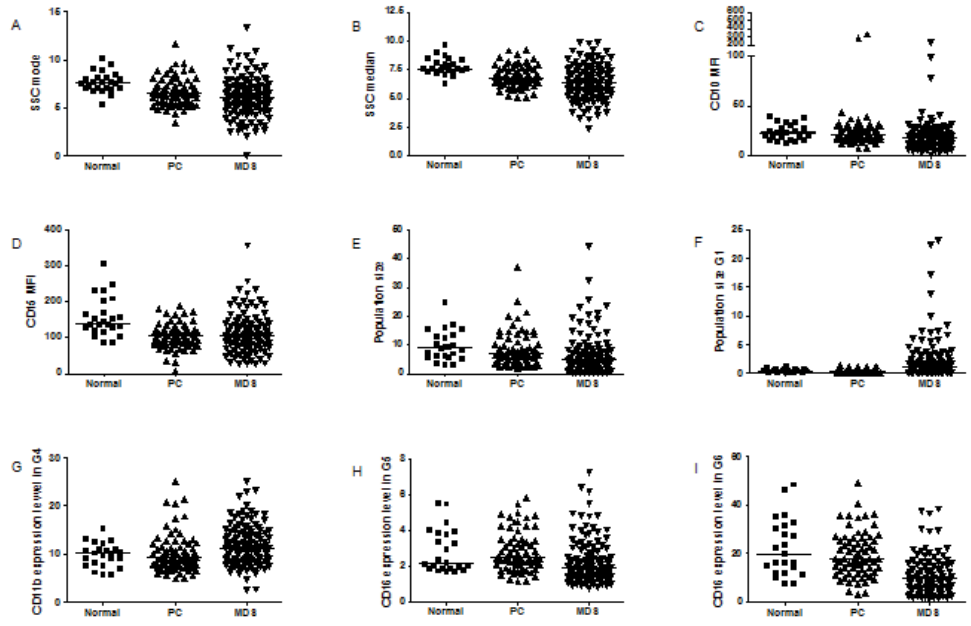
Table 2 (continue)

Parameter	Cell subset	MDS/HC	MDS/PC	HC/PC	Total
	CD7	ns	ns	ns	ns
	CD56	<0.001	0.001	0.004	<0.001
Population size§	Percentage	<0.001	<0.001	ns	<0.001
	Neutrophils/ lymphocytes	<0.001	<0.001	ns	<0.001
	Neutrophils/ monocytes	0.023	0.043	ns	0.021
	Percentage in G1	<0.001	<0.001	ns	<0.001
	Percentage in G2	ns	ns	ns	ns
	Percentage in G3	<0.001	<0.001	ns	<0.001
	Percentage in G4	ns	0.010	ns	0.021
	Percentage in G5	<0.001	0.001	ns	<0.001
	Percentage in G6	ns	ns	ns	ns
	Percentage in G7	ns	0.816	ns	ns
CD11b	MFI in G4	0.025	0.003	ns	0.003
	MFI in G5	ns	0.002	0.003	0.002
	MFI in G6	ns	0.002	0.027	0.004
	MFI in G7	ns	0.031	ns	ns
CD13	MFI in G1	0.020	ns	0.008	0.032
	MFI in G2	0.030	ns	ns	0.050
	MFI in G3	ns	<0.001	0.003	<0.001
	MFI in G4	ns	<0.001	0.014	<0.001
	MFI in G5	ns	0.007	0.002	0.003
	MFI in G6	0.014	ns	0.004	0.018
	MFI in G7	ns	ns	ns	ns
CD16	MFI in G5	0.010	<0.001	ns	<0.001
	MFI in G6	<0.001	<0.001	Ns	<0.001
	MFI in G7	<0.001	0.005	0.036	<0.001
Eosinophils	Percentage	ns	ns	ns	ns
Apoptosis	Percentage	<0.001	ns	<0.001	<0.001

An overview of the statistical results per flow cytometry parameter. *Calculated based on limited number of cases, as CD64 was only evaluated in small subset. §Percentage of neutrophils (as ratio to lymphocytes) within the white blood cell fraction, and percentages of cells gated within the granulocytic cell subset after exclusion of eosinophils and apoptosis. Abbreviations: SSC is sideward light scatter; MFI is median fluorescence intensity; G1-G7 are subsets that categorize seven different maturation stages within the granulocyte fraction defined by expression of CD117/ HLA-DR/CD13/CD11b/CD16 (**Figures S1 and S3**).

The SSC was also decreased in pathological controls but still significantly different from MDS (**Figure 1)**. The applied analysis software provides the median and the mode (peak channel value) of the SSC; when calculated for the total neutrophil population there was no difference between the mode or median. The SSC of the CD10-negative neutrophil compartment can be used to bypass the effect of hemodilution on FC results.[12] However, we showed that both the mode as the mean SSC value of the CD10-negative cell population did not differ significantly between MDS and pathological controls. The SSC mode and median values of the CD10-positive neutrophil compartment were significantly different between MDS and both control groups. These results are in line with the cytomorphological observation of hypogranulation of mature neutrophils in peripheral blood smears. Furthermore, as CD10 can be aberrantly lost on neutrophils in MDS patients this advocates against this strategy of neutrophil selection based on CD10.

Figure 1 Scatter plots of most discriminatory FC-parameters in MDS analysis.



The parameters that were highly significant different between groups (healthy, PC, MDS) are plotted. The lines represent the median value per patient group. SSC mode and median are decreased in MDS, CD10 MFI is decreased in MDS, CD15 MFI is decreased in MDS, total population size (ratio lymphocytes/granulocytes) is decreased in MDS, population size in G1 is increased in MDS (% within granulocytes), CD11b is increased in G4 in MDS, and CD16 is decreased in G6 in MDS. Note, that SSC, CD15-MFI and total population size are not MDS-specific as they are also decreased in pathological controls.

Maturation patterns

With use of differential expression of CD117, HLA-DR, CD11b, CD13 and CD16 we defined seven neutrophil maturation subsets (**Supplementary files**). An increased percentage of the most immature cells (G1: CD117-positive/HLA-DR-positive/CD11b-negative/CD13-positive/CD16-negative) revealed to be highly specific for MDS ($p<0.001$ for MDS versus both control groups). Furthermore, this G1 subset was significantly increased in MDS patients with >5% myeloid progenitors compared to MDS patients with <5% ($p<0.001$). Sizes of other immature neutrophil subsets were also significantly enlarged (G3) or decreased (G5) in MDS compared to the control groups.

We next evaluated CD11b-MFI, CD13-MFI, and CD16-MFI within the different maturational subsets. CD11b is normally present from G4 onwards, and CD16 is normally present from G5 onwards. In MDS patients, there was a significant increased CD11b-MFI in G4 (**Figure 1G**; N=24/123 MDS patients; $p=0.003$). CD16-MFI was significantly decreased in G5 and G6 (**Figure 1H-I;** N=17/123 MDS patients; $p<0.001$ and $p<0.001$). Early increase in expression of CD11b (G4) and delayed or low expression of CD16 (G5-G6) appeared to be highly specific for MDS. This delayed expression of CD16 resulted in the characteristic convex CD13/CD16 pattern (**Figure S3**).

CD10 is expressed on the most mature neutrophil subsets (from G6 onward). We found a significantly different expression of CD10 between the 3 groups. Remarkably, this was based on an increased CD10-MFI in pathological controls. Mainly caused by two outliers in the pathological control group. There was no difference with respect to CD10 expression between MDS and normal controls (G7; **Figure 1C**). Notably, CD10 is also lost on apoptotic cells. Apoptosis of hematopoietic cells within the bone marrow is a well-known phenomenon in MDS. Neutrophil apoptosis can easily be identified in the CD16/CD11b plot by selecting the CD16 diminished-to-positive, CD11b diminished cell population (**Figure 2**) (12). We found an increased percentage of apoptosis in MDS cases, however, there was not a significant difference between MDS and pathological controls.

CD15 is strongly expressed on mature granulocytes, with lower expression on the more immature cells. A decreased CD15-MFI is thought to be MDS-associated, in line with the left-shift commonly seen in MDS. In this analysis, a decreased CD15-MFI significantly distinguished MDS from healthy controls, but not from pathological controls.

Usually HLA-DR is not expressed on the more mature neutrophils in normal hematopoiesis. However, HLA-DR is described to be aberrantly expressed on neutrophils in MDS.[10] In this study the HLA-DR expression was not significantly different in MDS patients comparted to the control groups.

Lineage infidelity markers

CD14 may be expressed on neutrophils in MDS.[6] However, in our current cohort aberrant CD14 expression was not observed. Likewise, no expression of CD2, CD5, and CD7 was observed. In contrast, CD56 expression was detected on neutrophils in MDS and pathological controls, whereas it was absent on neutrophils in healthy controls (<1%). Thirty-seven percent of the MDS patients, 10% of the pathological control cases and none of the healthy controls had >1% CD56 positive neutrophils When applying a cut-off of ≥10%, 17% of the MDS patients and none of the control patients would be considered CD56-positive. In case of CD56-positive neutrophils ≥10%), monocytes also expressed CD56.

Other markers

There was no significant difference in percentage of CD64, percentage of CD71, or CD45-MFI between MDS and the control group.

Due to polymorphism of CD33, CD33 was analyzed in a group comparison (expression of CD33 on myeloid progenitors, neutrophils and monocytes). Regardless of this, CD33 expression was increased in 10 cases (8 MDS patients and 1 pathological control), decreased in 5 cases (3 MDS patients and 2 pathological controls) and normal in 158. This did not lead to a significant difference between the evaluated patient groups.

Identified parameters in an additional patient cohort

As described in the methods section, the aim of this study was to identify those parameters which are most significantly different between MDS, pathological controls and normal BM), especially focusing on markers that are significantly different between the MDS and pathological controls. To test the parameters identified in the study cohort, a test cohort of 70 MDS patients was assembled (**Table 1**). Within this validation cohort we tested, the percentage of cells within G1, CD11b expression in G3, CD16 expression in G4-G5, and the presence of ≥10% of CD56-positive neutrophils. Here, we found that 49 out of the 70 (70%) patients showed ≥1 of these aberrancies (results in Table S1). The 26 patients without any aberrancy were: 1 RA patient, 2 RARS, 9 RCMD patients (of which 4 had dysgranulopoiesis by cytomorphology), 6 RCMD-RS patients (of which 2 had dysgranulopoiesis by cytomorphology), 1 RAEB-1 patients, 2 RAEB-2 patients (both dysgranulopoiesis by cytomorphology), and 1 hypoplastic MDS patient.

In the test cohort, 41 of the 70 patients showed dysgranylopoiesis by cytomorphology, and 49 patients of the 70 patients had neutrophil aberrancies according to the described FC parameters. Although both techniques describe different cell features, there was a significant correlation between these results ($P=0.02$).

Discussion

The granulocytic cell differentiation forms the corner stone of currently applied diagnostic strategies for myelodysplastic syndromes (MDS). The aim of this study was to provide a rational for reducing the number of parameters in current MDS-FC panels, to implement the wider use of FC in MDS diagnosis. In daily clinical care, patients with possible MDS will present based on the presence of cytopenias. Therefore, to optimize the use of MDS-FC parameter selection focus should be on distinction between MDS and pathological controls, and not on the distinction of patients from normal, healthy bone marrow per se. The data show that an increase in the percentage of the most immature neutrophil population (assigned as G1), the early expression of CD11b (G1), decreased expression of CD16 on maturing neutrophils (G5-G6; convex CD13/CD16 pattern), and the expression of CD56 (10% cut-off) were highly indicative for MDS. Within our cohort the sideward light scatter (SSC; as a ratio to lymphocyte SSC) proved to be frequently decreased in MDS. This confirms data from Ogata and colleagues who described SSC as a cardinal diagnostic parameter.[14] The SSC might be of additional relevance but needs to be interpreted with caution, as it distincts MDS from healthy controls but not from pathological controls. In our data SSC is as frequently decreased in pathological controls. Therefore, in this analysis the SSC is not included in validation cohort.

Total percentage of neutrophils and the CD10-MFI are also markers that might be of additional relevance, but one needs to keep in mind that these are easily influenced by sample quality as they are influenced by hemodilution.

Abnormal maturation patterns reflected by abnormal CD11b/CD13, CD13/CD16 and CD11b/CD16 patterns are also described in pathological controls.[15] However, early increased expression of CD11b and decreased expression of CD16 appeared to be highly indicative for MDS. Note, that CD16 detects a glycosylphosphatidylinositol-linked antigen and loss can also be characteristic for a paroxysmal nocturnal hemoglobinuria (PNH) clone, co-occurring with MDS (seen in very low frequencies) and aplastic anemia. However, the difference between PNH and MDS is easily made by total absence of CD16 versus decreased expression on only the most mature cell subsets.

Another pitfall in the selection of G1-G7 subgroups by pattern recognition is the asynchronous left shift described in MDS. Furthermore, aberrant expression of subpopulation defining markers can influence the population selection, i.e. prolonged expression of CD117 that is therefore still present in G3. Here, expression of CD45 and SSC might help to correctly identify the different subpopulation. For G1 this forms less of a problem, as this population can easily be identified by CD34negative/CD117positive and the previous described markers (**Supplementary files**).

Increased apoptosis of BM cells is one of the main features in low grade MDS bone marrow. Since, apoptosis is influenced by sample quality and processing time, it may not be a reliable diagnostic parameter.[4] Our data showed that increased apoptosis was not MDS-restricted. However, it remains important to identify apoptosis as apoptosis can result in altered marker expression, i.e. decreased CD10, CD11b and CD16. Marker aberrancies that might therefore be falsely interpreted as typical MDS.

Finally, a considerable percentage of CD56-positive neutrophils (≥10%) were only detected on neutrophils of MDS patients. These results were in line with Bardet et al.[16] However, they pointed out that neutrophil-CD56 expression was not a sensitive marker as only 3% of the MDS cases showed CD56-positive neutrophils (cut-off >30%). In our analyses, none of the pathological controls had ≥10% CD56-positive neutrophils. Therefore, we propose that CD56-positive neutrophils are highly suggestive for MDS, and a threshold of ≥10% is feasible to score CD56 as an aberrant marker.

Our data on flow cytometric analysis of dysplastic granulopoieis underscore the contribution of FC in the distinction of patients with cytopenia based on MDS or other causes of cytopenia. Therefore, FC is currently part of the recommendations for the diagnostic work-up in MDS. However, the use of FC in MDS needs further refinements and is subject of extensive research to further improve MDS diagnosis.[2] Analysis of granulopoiesis is an important part of current MDS-FC strategies. We identified herein that only part of the recommended markers is highly indicative for MDS. This is the first step to optimize more concise MDS-FC panels that are more specific, less time-consuming and easier to apply in routine daily laboratory medicine.

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Conflict of interest statement: none declared.

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Supplementary files

Figure S1 The evaluated neutrophil maturation stages.
The graph illustrates the median CD-marker expression levels of 20 normal controls for each maturation stage, from most immature (G1) to most immature (G7). The expression levels are in logarithmic scale. Additionally, HLA-DR and CD117 expression were used.

G1: CD117 positive/HLA-DR positive/CD11b negative/CD13 bright/CD16 negative
G2: CD117 positive/HLA-DR negative/CD11b negative/CD13 positive/CD16 negative
G3: CD117 negative/HLA-DR negative/CD11b negative/CD13 negative/CD16 negative
G4: CD117 negative/HLA-DR negative/CD11b positive/CD13 negative/CD16 negative
G5: CD117 negative/HLA-DR negative/CD11b positive/CD13 negative/CD16 positive
G6: CD117 negative/HLA-DR negative/CD11b positive/CD13 positive/CD16 positive
G7: CD117 negative/HLA-DR negative/CD11b positive/CD13 bright/CD16 bright

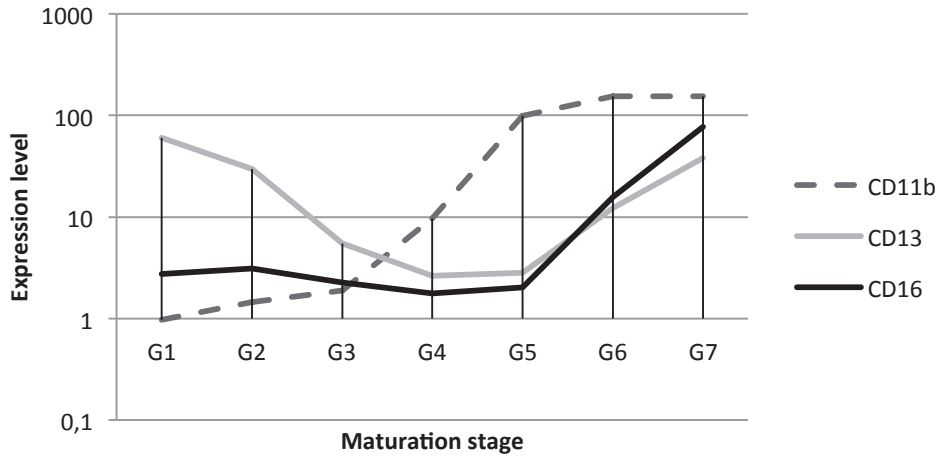

Figure S2 Example MDS neutrophil maturation stages.
This graph illustrates the expression levels of CD11b, CD13 and CD16 of the same MDS patient as provides in **Figure S3**. CD11b is normally expressed within the different subsets. CD13 is increased expressed in G1-G3 and stays expressed, even in the normally CD13 negative population (G4). CD16 is decreased expressed in G5-G6.

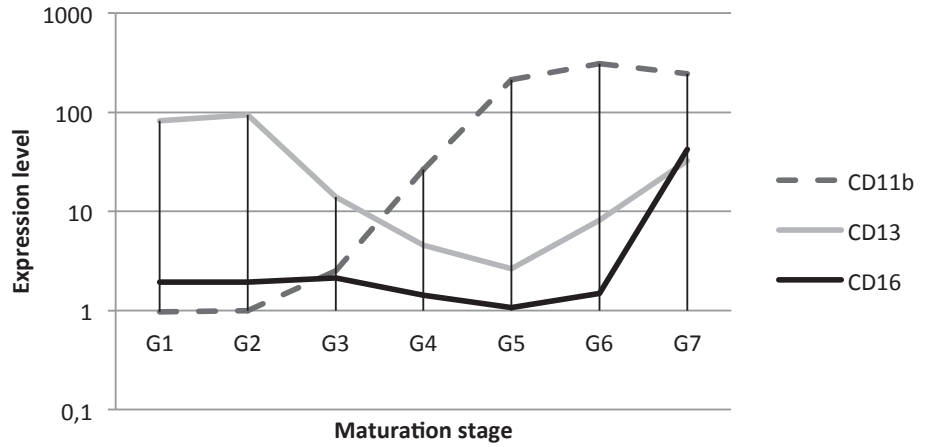

Table S1 Results of the test cohort.

WHO2001	%G1	CD11bG3	CD16G4-5	CD56	Total
RA	1	0	0	0	1
RA	0	0	1	0	1
RA	0	0	0	0	0
RARS	0	0	0	0	0
RARS	0	0	0	0	0
RCMD	1	0	0	1	2
RCMD	1	0	1	0	2
RCMD	1	1	0	0	2
RCMD	0	0	1	0	1
RCMD	0	0	0	0	0
RCMD	0	0	0	0	0
RCMD	0	0	0	0	0
RCMD	0	0	0	0	0
RCMD	0	0	0	0	0
RCMD	0	0	0	0	0
RCMD	0	0	0	0	0
RCMD	0	0	0	0	0
RCMD	1	0	1	1	4
RCMD	1	0	1	0	3
RCMD	0	0	1	0	2
RCMD	1	0	1	0	2
RCMD	0	1	1	0	2

Table S1 (continue)

WHO2001	%G1	CD11bG3	CD16G4-5	CD56	Total
RCMD	1	0	1	0	2
RCMD	1	0	0	0	2
RCMD	0	0	0	0	1
RCMD	0		1	0	1
RCMD	0	0	0	0	0
RCMD	0	0	0	0	0
RCMD-RS	1	0	1	1	3
RCMD-RS	0	0	1	1	2
RCMD-RS	1	0	1	0	2
RCMD-RS	1	0	0	0	1
RCMD-RS	1	0	0	0	1
RCMD-RS	0	1	0	0	1
RCMD-RS	0	0	1	0	1
RCMD-RS	0	1	0	0	1
RCMD-RS	0	0	0	0	0
RCMD-RS	0	0	0	0	0
RCMD-RS	0	0	0	0	0
RCMD-RS	0	0	0	0	0
RCMD-RS	0	0	0	0	0
RCMD-RS	1	0	1	0	2
RCMD-RS	0	0	0	0	1
RCMD-RS	1	1	0	0	1
RCMD-RS	0	0	0	0	1
RCMD-RS	0	1	1	0	1
RAEB-1	1	0	1	0	2
RAEB-1	1	0	1	0	2
RAEB-1	1	0	0	0	1
RAEB-1	0	0	0	0	0
RAEB-1	1	0	1	1	3
RAEB-1	1	0	1	0	3
RAEB-1	1	0	1	0	3
RAEB-1	1	0	0	0	1
RAEB-1	1	0	0	0	1
RAEB-2	1	0	1	0	2
RAEB-2	1	0	1	0	2
RAEB-2	1	0	0	1	2
RAEB-2	1	0	0	0	1
RAEB-2	0	0	1	0	1
RAEB-2	0	0	1	0	1
RAEB-2	1	0	1	1	3
RAEB-2	1	0	1	0	3
RAEB-2	0	0	1	0	3
RAEB-2	0	0	1	0	1
RAEB-2	0	0	0	0	0
RAEB-2	1	1	1	0	3
Del(5q)	1	0	0	0	1
Hypoplastic MDS	0	0	0	0	0
Hypoplastic MDS	1	0	0	0	1

CHAPTER 4

IMMUNOPHENOTYPIC ANALYSIS OF ERYTHROID DYSPLASIA IN MYELODYSPLASTIC SYNDROMES

A REPORT FROM THE IMDSFLOW WORKING GROUP

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HAEMATOLOGICA. 2017; 102: 308-319

Abstract

Current recommendations for diagnosing myelodysplastic syndromes (MDS) endorse flow cytometry (FC) as an informative tool. Most FC protocols focus on analyzing progenitor cells and the maturing myelomonocytic lineage. However, one of the most frequently observed symptoms in MDS is anemia, which may be associated with dyserythropoiesis. Therefore, analysis of changes in FC features of nucleated erythroid cells may complement current FC tools. The multicenter study within the IMDSFlow Working Group reported herein focused on defining FC parameters that enable discrimination of dyserythropoiesis associated with MDS from non-clonal cytopenias. Data from a learning cohort were compared between MDS and controls, the results were validated in a separate cohort. The learning cohort comprised 253 MDS cases, 290 pathological and 142 normal controls; the validation cohort 150 MDS cases, 153 pathological and 49 normal controls. Analysis of expression of CD36 and CD71 in combination with the percentage of $CD117^+$ erythroid progenitors provided the best discrimination between MDS and non-clonal cytopenia (specificity and sensitivity: 90% and 33% in the learning cohort and 92% and 24% in the validation cohort, respectively). This marker combination may improve the evaluation of cytopenic cases with suspected MDS, particularly when combined with FC assessment of the myelomonocytic lineage.

Introduction

Discriminating between cytopenia due to myelodysplastic syndromes (MDS) and cytopenia due to other (non-clonal) causes can be challenging, especially when dysplasia as assessed by cytomorphology does not fulfill the diagnostic criteria of MDS according to WHO criteria, and when other MDS-specific features are absent (e.g. ring sideroblasts (RS) or cytogenetic aberrations). Current recommendations for the diagnosis of MDS endorse flow cytometry (FC) as a valuable additional diagnostic tool. In this respect, it has been recommended to follow the guidelines set down by the International/European LeukemiaNet Working Group for FC in MDS (IMDSFlow).[1-3]

Despite the fact that FC for MDS correlates with cytomorphology, the sensitivity of current validated FC scores for diagnosing MDS requires improvement.[4-7] So far, most of the designed FC scores have comprised the analysis of the (im) mature myelomonocytic lineage with a median sensitivity of 75% for identifying MDS (median specificity, 94%; summarized in [3]). Since anemia is a frequently observed symptom in MDS, often accompanied by erythroid dysplasia, analysis of immunophenotypic changes of nucleated erythroid cells (NEC) may complement current FC analysis.[8, 9] Thus far, this has not been studied in great detail. Integration of results from analysis of the erythroid lineage to the primarily myelomonocytic and progenitor cell-based FC scores may improve sensitivity of FC analysis in MDS.[7, 10-12]

Incorporating erythroid markers in FC protocols requires knowledge of normal erythroid differentiation, and of potential aberrancies and pitfalls. The characteristic morphological stages of normal erythroid differentiation are reflected by their light scatter properties and by their differential expression of CD45, CD117, CD105, CD71, CD36 and/or CD235a (**Figure 1**).[13-15] Some of the FC aberrancies that have been reported to reflect MDS-related dyserythropoiesis are: **a)** an increased number of NEC within total nucleated cells; **b)** an altered proportion of consecutive erythroid differentiation stages, such as an increased number of immature erythroid cells ($CD117^+$ and/or $CD105^+$) or, by contrast, a decrease in erythroid progenitors; **c)** an abnormal pattern of CD71 versus CD235a; **d)** a reduced expression of CD71 and/or CD36; and **e)** an overexpression of CD105. Most of these aberrancies are present in 70–80% of MDS cases.[7, 10-12, 14, 16-20] However, a number of features may be shared across the spectrum of non-clonal cytopenias.[21-23]

The multicenter study reported herein focused on defining those erythroid FC parameters that enable discrimination of dyserythropoiesis associated with MDS from non-clonal cytopenias. Hereto, data from a learning cohort were compared between MDS patients and controls and the results were validated in a separate cohort.

Material and methods

MDS patients and controls

Nineteen centers (members of the IMDSFlow group) collected FC data on the erythroid lineage in mainly low grade MDS cases (<5% blasts) and controls. Data were acquired from bone marrow samples taken from 1037 patients and healthy controls after informed consent in accordance with the Declaration of Helsinki; where required, local ethics committee approval was obtained. The learning cohort comprised 685 cases (18 centers, data collected between October 2012 and September 2013) and the validation cohort 352 cases (9 centers, data collected between December 2013 and April 2014). In total 403 MDS cases, 443 pathological controls, and 191 normal controls were analyzed (**Tables 1 and 2**). Information regarding age, gender, cytomorphology and cytogenetics were requested. One center with limited access to cytomorphology results only included MDS patients with typical features of MDS as the presence of more than 15% RS and MDS-

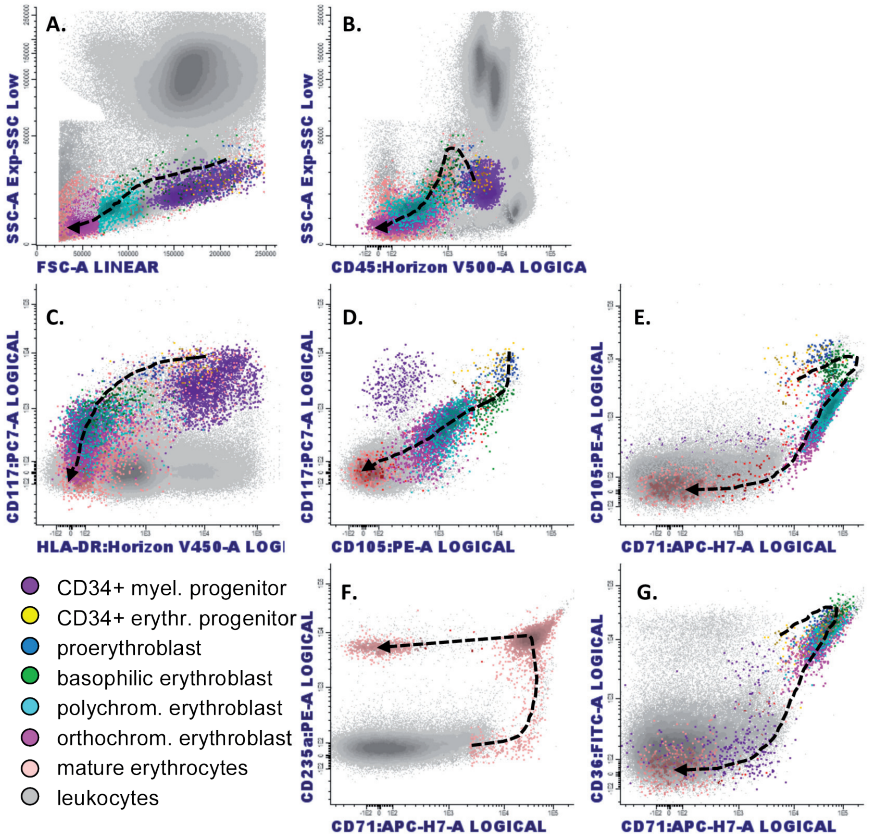


Figure 1 Flow cytometric profiles of normal erythroid differentiation.
Early erythroid precursors are defined as $CD45^{dim}/SSC^{int}/CD34^{+}/CD117^{+}/CD105^{+}$, proerythroblasts as $CD45^{dim/-}/SSC^{int}/CD117^{+}/CD105^{+}/CD71^{+}/CD36^{+}/CD235a^{+}$, basophilic erythroblasts as $CD45^{dim/-}/SSC^{int}/CD105^{+}/CD36^{++}/CD71^{+}/CD235a^{+}$, polychromatic erythroblasts as $CD45\text{-}/SSC^{low}/FSC^{int}/CD36^{+}/CD71^{+}/CD235a^{+}$ and orthochromatic erythroblasts $CD45^{-}/SSC^{low}/FSC^{low}/CD36^{+/-}/CD71^{+}/CD235a^{+}$. Indicated colors reflecting erythroid subsets are not present in the CD71 vs. CD235a plot. Pink colored cells represent the total erythroid lineage in this plot. Mature erythrocytes ($CD45^{-}/CD36^{-}/CD71^{-}/CD235a^{++}$) can be seen in improperly lysed cell preparations (Fig 1F). Reticulocytes are not covered in these graphs, but they may appear as $CD71^{dim\text{-}to\text{-}negative}$ in non-lysed cell preparations. Myeloid progenitors are $CD34^{+}/CD117^{+}/HLA\text{-}DR^{+}/CD105^{-}$ (Figure 1C and D.) and have slightly higher CD45 expression than erythroid precursors; moreover, erythroid cells do not express HLA-DR in contrast to myeloid progenitors (adapted from references (13-15))

Table 1 Characteristics of MDS patients and controls in the learning and validation cohort

	Learning cohort	Validation cohort
Normal		
n	142	49
age	60 (20-86)[a]	64 (24-86)[a]
male:female	1.4:1	2.3:1
Pathological controls		
n	290	153
age	62 (18-92)[a]	72 (20-98)[a]
male:female	1:1	1.2:1
MDS (non-RAEB cases)		
n	245	129
age	72 (23-94)[a]	75 (40-95)[a]
male:female	1.1:1	1.6:1
MDS-RAEB		
n	8	21
age	61 (43-85)[a]	79 (39-88)[a]
male:female	1:1	2.5:1
Comparison of age		
normal vs. pathological controls	p = 0.007	p = 0.193
pathological controls vs. MDS	p < 0.001	p = 0.013
Comparison of gender	n.s.	n.s.

Note: [a]data is expressed as the median and range; abbreviations: MDS: myelodysplastic syndromes; n: number; n.s.: not significant, RAEB: refractory anemia with excess of blasts.

associated cytogenetic anomalies. In 354/403 MDS cases subclassification according to the WHO-2008 (ref. 24) was provided. MDS-RAEB (refractory anemia with excess of blasts; 29/403 MDS) was considered a separate subset, because it is less challenging to separate these MDS cases from pathological controls than low grade MDS. The median contribution per center to the total study cohort was 47 cases (range 6–100) and the median number of erythroid FC markers analyzed per case was 7 (range 1–9 of 10 proposed markers).

Sample preparation and antibody combinations

Flow cytometric analysis in MDS requires the removal of mature, enucleated erythrocytes through the use of lysis protocols. The vast majority of centers used ammonium chloride-based solutions, either home-made or commercial (e.g. PharmLyse; BD Biosciences, San Jose, CA); two centers used FACSLyse (BD Biosciences) and one other VersaLyse (Beckman Coulter, Miami, FL). FACSLyse contains a fixative, whereas VersaLyse is recommended for use with a fixative when the sample contains anticoagulants other than EDTA. The lysis time and temperature varied among centers (5-25 minutes and 4-37°C, respectively), but most lysed for 10 minutes (n=10) at room temperature (n=16). Two centers reported the use of an additional fixative in their staining protocols, both in combination with an ammonium chloride-based lysing solution. Detailed information can be found in **Supplementary information**. Most centers used the IMDSFlow-recommended lyse-stain-wash procedure; five centers performed stain-lyse-wash. Antibody combinations were similar between centers, but clones and fluorochromes differed. Most centers used a backbone of CD45 and CD34 and/or CD117 and added antibodies such as CD235a, CD71, CD36 and CD105. Examples of antibody combinations and panels were described previously.[2, 25, 26] Nuclear dyes were not routinely included in the panels; one center applied the live/dead stain 7-AAD. The flow cytometers used included: FACSCalibur (BD Biosciences; n=3); FACS CANTO-II (BD Biosciences; n=10); a combination of FACSCalibur and FACS CANTO-II (both BD Biosciences; n=2); and Navios (Beckman Coulter; n=4). Panels comprised 4-, 5-, 6-, 8- and/or 10-color FC; WinList 7.0 (Verity Software House, Topsham, ME), Kaluza (Beckman Coulter), CellQuestPro, FACS-DIVA (both BD Biosciences), and/or Infinicyt (Cytognos, Salamanca, Spain) software packages were used for data analysis.

Table 2 Subcategories of MDS and pathological controls in the learning and validation cohort

	Learning cohort	Validation cohort
MDS subcategories		
RCUD	23 (9.1%)	14 (10%)
RARS	16 (6.3%)	12 (9.3%)
RARS-t	2 (0.8%)	1 (0.7%)
RCMD	155 (61.2%)	75 (50%)
del(5q)	14 (5.5%)	5 (3.3%)
MDS-U	3 (1.2%)	3 (2.0%)
RAEB-1	5 (2.0%)	12 (8.0%)
RAEB-2	3 (1.2%)	9 (6.0%)
other		2 (1.3%)*
not specified	32 (13%)	17 (11%)**
Subcategories of pathological controls		
iron deficiency anemia	22 (7.6%)	13 (8.5%)
iron incorporation disturbances or anemia in chronic disease	42 (14.5%)	8 (5.2%)
vitamin B12/folic acid deficiencies	11 (3.8%)	11 (7.2%)
anemia in auto-immune diseases	13 (4.5%)	7 (4.6%)
anemia due to renal failure	6 (2.1%)	5 (3.3%)
anemia other	9 (3.1%)	9 (5.9%)
cytopenia associated with marrow infiltration	20 (6.9%)	7 (4.6%)
cytopenia induced by chemotherapy or medication or post-SCT	27 (9.3%)	8 (5.2%)
ITP or neutropenia or auto immune cytopenia NOS	30 (10.3%)	14 (9.2%)
reactive conditions or cytopenia induced by infections	32 (11%)	10 (6.5%)
normal bone marrow, peripheral cytopenia	6 (2.1%)	0
other than defined subcategories	29 (10%)	23 (15%)
inconclusive	11 (3.8%)	8 (5.2%)
non clonal cytopenia NOS	25 (8.6%)	19 (12,4%)
ET, PV, primary myelofibrosis	5 (1.7%)	7 (4.6%)
PNH	1 (0.3%)	0
AA	1 (0.3%)	4 (2.6%)

Values in brackets represent the relative distribution within MDS or pathological control subgroups; *other concerns one case of hypoplastic MDS and one case of MDS with fibrosis; **not specified, but the presence of MDS-associated cytogenetic aberrations indicated; abbreviations: AA: aplastic anemia, ET: essential thrombocythemia; MDS: myelodysplastic syndromes, MDS-U: MDS-unclassifiable; NOS: not otherwise specified, PNH: paroxysmal

hemoglobinuria, PV polycythemia vera; RA: refractory anemia, RAEB: refractory anemia with excess of blasts, RCMD: refractory cytopenia with multilineage dysplasia, RCUD: refractory cytopenia with unilineage dysplasia, del(5q): MDS with isolated del(5q). Comparison of the distribution of MDS subsets among the learning and validation cohorts did not differ (Chi-Square test; p=0.511 when excluding MDS RAEB cases); the distribution of subsets of pathological controls did (p<0.001).

Gating strategy and data collection

The gating strategy was discussed during the IMDSFlow meeting in 2011 and re-evaluated in 2012. All participants performed FC analysis of the erythroid lineage defined as $CD45^{dim\text{-}to\text{-}negative}$ and $SSC^{low\text{-}to\text{-}intermediate}$. Noteworthy, the initially proposed gating strategy (erythroid lineage defined by CD45 negativity) was altered to include early erythroid precursors that are within the $CD45^{dim}$ cell population.[2, 3, 12] Six or more color panels enabled the inclusion of a myeloid-defining marker such as CD13 or CD33 and a more accurate separation of myeloid and erythroid progenitors. Moreover, to exclude platelets and platelet aggregates, a combination of scatter properties and $CD36^{high}/CD71^{-}$ was suggested. The final gating strategy was distributed among all centers (further details in **Supplementary information** on gating strategy).

The following parameters were collected: the percentage of NEC within all nucleated cells; the expression pattern of CD71 versus CD235a; the percentage of $CD71^{dim}CD235a^{+}$ cells within the CD71/CD235a pattern; CD71 and CD36 expression levels; the percentage of $CD117^{+}$ cells in the erythroid compartment; CD105 expression level and the percentage of $CD105^{+}$ cells in the erythroid compartment. Recent knowledge, such as the finding that CD71 and CD36 expression represented as CV is statistically more significant than when represented as mean fluorescence intensity (MFI)[27], led to adjustments in the initially proposed protocol and hence, reanalysis of the list mode data files by the individual centers. Some examples of FC plots of MDS in comparison to normal subjects are displayed in **Supplementary information** on gating strategy.

Statistical analyses

Due to differences in sample processing, instrument settings, clones, and fluorochromes between centers, the expression levels of CD71, CD36, and CD105 varied. Therefore, the median expression levels of CD36, CD71, and CD105 in the subset of normal bone marrow samples were calculated for each individual center. Expression levels were then normalized against the median value for that particular marker for each center separately. Patient and control groups were compared using the Kruskal Wallis test for continuous data, and the Chi-square or the Fisher's exact test for dichotomous data. Correlations between

certain markers, and between markers and age, were analyzed using Spearman's rank correlation coefficients. Cut-off values for aberrancies were based on the 10^{th} and/or 90^{th} percentile of the data of pathological controls in the learning cohort. Multivariate logistic regression analyses were performed to determine the erythroid markers that discriminate between pathological controls and MDS; all variables that displayed a univariate difference of p<0.1 were included in a backward selection procedure based on the Likelihood Ratio score. Regression coefficients of the variables in the final model were used to define the weight of these markers in a descriptive score for dyserythropoiesis. Cut-off level of the score indicating MDS-associated erythroid aberrancies was determined based on the total weight of these variables and a specificity of at least 90%. The sensitivity and specificity of the marker combination were calculated to illustrate predictive accuracy. The data were analyzed using SPSS 20.0 (IBM Corp, Armonk, NY), and GraphPad 6.0 software (La Jolla, CA). P-values <0.05 were considered significant.

Results

Flow cytometric analysis of the erythroid lineage in normal bone marrow samples: comparison of results from participating centers

Discrepancies in erythroid analysis between centers (and samples) can occur at several levels: **a)** sample quality (e.g., hemodilution); **b)** sample preparation (e.g., lysing procedure); **c)** data acquisition (e.g., acquisition rate and threshold of forward light scatter); and **d)** degree of adherence to the proposed gating strategy. Therefore, we first compared the FC results for normal bone marrow samples (learning cohort) between centers in terms of each defined marker. The percentage of NEC was highly diverse among centers; yet, it seemed to be independent of the applied lysing method (**Supplementary Figure 1**). Similarly, the percentage of $CD71^{dim}$ cells differed largely between centers. Furthermore, two centers reported higher percentages of $CD117^{+}$ erythroid progenitors (up to 50% within the NEC) than the other centers (<15%). Results for one center could be explained by their stringent lysing procedures (i.e. 15 minutes at 37°C) which removed more mature (orthochromatic and polychromatic) erythroblasts resulting in a relative increase in early progenitors (data not shown). To circumvent the issues regarding differences in percentages of erythroid (sub)populations between centers, data of percentages of NEC, $CD117^{+}$ and $CD105^{+}$ erythroid progenitors were also normalized as described for expression levels of the antigens (see Material and Methods statistics section); these are further referred to as relative percentages, i.e. relative to the median percentage in normal bone marrow samples (**Supplementary Figure 2**). $CD71^{dim}$ cells are rarely seen

in normal controls; therefore, results for the percentage CD71^{dim} could not be normalized.

Erythroid aberrancies that may discriminate between MDS and pathological controls

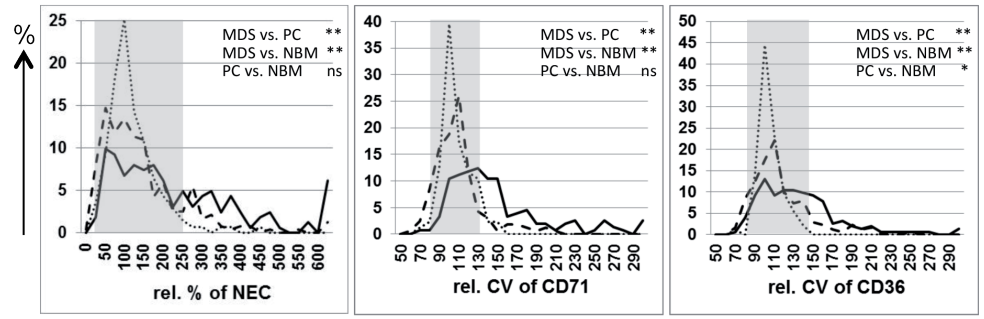
Next, all FC-erythroid parameters in the learning cohort were compared between MDS and controls. The results from the learning cohort are summarized in **Figure 2** (p-values in **Supplementary Table 1**). The relative percentage of NEC within the total nucleated cell population was significantly higher in MDS than in the pathological and normal controls (p<0.001). Similarly, the CD71-CD235a differentiation pattern was more frequently considered aberrant in MDS (65%, 109/167 cases) than in pathological and normal controls (18% (44/254) and 3.7% (5/134) of cases, respectively (p<0.001)). To objectify the evaluation of this pattern, we analyzed its components separately. CD71 expression was analyzed in terms of MFI, CV, and the presence of a subpopulation with reduced CD71 expression (CD71^{dim}). The relative CD71 MFI was significantly reduced in MDS, whereas the relative CV for CD71 and the percentage of CD71^{dim} cells were significantly higher than those in both control groups (p<0.001); no significant differences were observed between the pathological controls and normal bone marrow samples. The expression of CD235a largely depends on the success of removing mature erythrocytes from a sample. Moreover, membrane fragments of lysed erythrocytes may stick to other cells in the analysis sample mimicking positivity. Hence, this parameter was considered too unreliable for evaluation when considered individually.

The percentage of immature erythroid progenitors can also affect the appearance of the differentiation pattern. This was evaluated by calculating the relative percentage of CD117^{+} (and CD105^{+}) erythroid progenitor cells, which revealed a broader range of these cells in MDS, although not significantly different from the control groups.

Relative expression of CD105 was either increased or decreased in MDS. Nonetheless, CD105 expression did not discriminate between MDS and pathological controls.

Similar to that for CD71, the relative MFI of CD36 was significantly lower and the relative CV for CD36 was significantly higher in MDS than in the control groups.

Figure 2 Distribution of results for erythroid markers analyzed by flow cytometry among MDS patients and controls within the learning cohort. Results of the analysis of indicated markers of the erythroid lineage are plotted along the X-axes: relative (rel.) percentages of nucleated erythroid cells (NEC), rel. mean fluorescence intensity (MFI) for CD36, CD71 and CD105, rel. coefficient of variation (CV) of CD36 and CD71, and rel. percentages of CD117^{+} and CD105^{+} erythroid progenitors. Relative frequencies (as percentage of the MDS or control cohort for a particular marker) are depicted along the Y-axes. Dotted lines represent results for normal bone marrow (NBM) samples, dashed lines pathological controls (PC) and solid lines MDS cases. P-values of comparison between groups are depicted: **: <0.001, *: <0.05, ns: not significant (Kruskal Wallis test). Grey boxes indicate reference ranges for the analyzed markers as defined by 10th and 90th percentiles of pathological controls. Note that MDS-RAEB cases were excluded from the MDS group in these graphs.



To summarize, the markers that showed a significantly different distribution in MDS as compared to controls were: the relative percentage of NEC and the percentage of CD71^{dim} cells (increased in MDS); the relative MFI of CD71 and CD36 (decreased in MDS); and the relative CV for CD71 and CD36 (increased in MDS); **Figure 2**.

Selection of a combination of erythroid FC aberrancies that distinguish MDS from pathological controls

To be applicable in FC analysis of a single patient in daily practice, cut-offs for the identification of MDS-associated changes of all potential aberrancies were defined (10th and 90th percentiles of the pathological controls, **Supplementary Table 2**) and compared between MDS and controls (**Table 3**). All parameters differed between groups at a p-value of <0.1 and thus could have been considered for the multivariate logistic regression analysis. However, due to large differences between centers, regarding the percentages of NEC and CD71^{dim} cells (likely due to technical variation as shown for normal controls), these parameters were not entered in the multivariate analysis. Besides, irrespective of the finding that data for CD105 significantly discriminated between subgroups (**Table 3**), this marker was not included. Entering CD105 data would have reduced the power of the (multicenter) analysis, since data on CD105 were only available in a limited number of centers (5/18) and cases.

Table 3 Aberrancies in FC markers of the erythroid lineage between MDS and controls within the learning cohort

	NBM	PC	MDS	p-value MDS vs. PC	p-value MDS vs. NBM	p-value PC vs. NBM
rel. %NEC	2.9	10.1	**32.1**	**<0.001**	<0.001	0.013
pattern CD71 vs. CD235a	3.7	17.3	**64.9**	**<0.001**	<0.001	<0.001
%CD71^{dim}	0.8	10.0	**31.5**	**<0.001**	<0.001	<0.001
rel. MFI of CD71	4.0	10.0	**27.8**	**<0.001**	<0.001	0.045
rel.CV of CD71	4.5	10.3	**45.5**	**<0.001**	<0.001	0.152
rel. MFI of CD36	0.8	10.3	**25.8**	**0.001**	<0.001	<0.001
rel. CV of CD36	0.0	10.2	**30.1**	**<0.001**	<0.001	0.001
rel. %CD117 progenitors	8.2	19.4	**33.8**	**0.005**	<0.001	0.008
rel. %CD105 progenitors	6.7	20.8	**48.4**	**0.001**	<0.001	0.092
rel. MFI of CD105	29.8	22.5	**58.7**	**<0.001**	0.004	0.395

Note: After applying cut-offs as defined in the set of pathological controls, the results were expressed as '0' and '1' for within and beyond reference range(s), respectively (ranges as displayed in *Supplementary table 2*). Percentages of subjects with aberrancy are displayed for normal bone marrow (NBM), pathological controls (PC) and MDS cases. Results were compared among subgroups using Fisher's Exact test; p-values are depicted. Abbreviations; CV: coefficient of variation; dim: diminished; MFI: mean fluorescence intensity; NEC: nucleated erythroid cells; rel.:relative. MDS-RAEB cases were excluded from this analysis.

The multivariate logistic regression analysis identified the CD36 CV as the best discriminator between MDS and pathological controls in combination with the CV of CD71, the MFI of CD71 and the percentage of CD117^{+} erythroid cells (**Table 4A**). These four markers were used to define a FC-erythroid dysplasia score in which aberrancies were scored in a weighted manner: four points for increase in CD36 CV; three points for increase in CD71 CV; two points for decreased CD71 MFI; and two points in case of decreased or increased percentage of CD117^{+} erythroid cells (reference ranges are summarized in **Table 5**). A cut-off of ≥5 points resulted in the identification of MDS-associated erythroid aberrancies by FC at a specificity of 90% (95% CI: 84–94%) and a sensitivity of 33% (95% CI: 24–42). Results for the selected markers and the application of the FC-erythroid dysplasia score in the learning cohort are displayed in **Table 6** and **Figure 3A**. In daily practice, a numerical score based on one point per aberrancy would be more convenient. This involves the definition of a new cut-off; i.e., ≥2 aberrant markers; **Figure 3C**. Note that, the exception to this numerical score is that the combination of aberrancies in CD71 MFI and percentage CD117^{+} alone is not sufficient to conclude dyserythropoiesis by FC (only 4 points in the weighted score). The latter was seen in one pathological control and three MDS cases.

Table 4 Results of multivariate logistic regression analysis in learning (A), validation (B) and combined cohorts (C and D).

	parameter	exp(B)	95% CI	p-value
A learning cohort	**CD36 CV**	3.7	1.6 – 8.5	0.003
	CD71 CV	3.2	1.6 – 6.4	0.001
	CD71 MFI	2.2	1.1 – 4.5	0.033
	%CD117	1.7	0.92 – 3.2	0.084
B validation cohort	**CD36 CV**	2.6	0.94 – 7.4	0.067
	%CD117	2.6	1.1 – 5.2	0.007
	CD36 MFI	2.3	1.3 – 4.9	0.038
C combined cohorts	**CD36 CV**	2.9	1.5 – 5.6	0.001
	CD71 CV	2.9	1.7 – 4.9	<0.001
	%CD117	2.0	1.3 – 3.2	0.004
	CD36 MFI	1.7	0.99 – 2.8	0.053
D combined cohorts	**CD71 MFI**	3.6	1.2 – 11	0.025
	%CD105	3.6	2.0 – 6.5	<0.001
	CD36 CV	3.2	1.1 – 9.7	0.036
	CD71 CV	3.1	1.4 – 7.1	0.007

Note: Panels A-C: markers entered in the analysis were relative CD36 MFI, CD36 CV, CD71 MFI and CD71 CV, and the relative percentage of CD117$^+$ erythroid cells (%CD117). Panel D: markers entered in the analysis were similar as in panel A-C but now including the percentage of CD105$^+$ erythroid cells (%CD105). Relative expression of CD105 was excluded because of a significant correlation with age.
Panel A; 270/535 cases were available for analysis of which 152 pathological controls and 118 MDS cases in the learning cohort; p<0.001). Panel B; 199/282 cases were available for analysis of which 106 pathological controls and 93 MDS cases; p<0.001). Panel C; 469/817 cases were available for analysis of which 258 pathological controls and 211 MDS cases; p<0.001). Panel D; 242/575 cases were available for analysis of which 125 pathological controls and 117 MDS cases; p<0.001).

Table 5 Reference ranges of FC parameters incorporated in the FC-erythroid dysplasia score

	Reference Ranges[c]	# of PC cases[a]	# of NBM cases[b]
relative CV of CD36	**<145%**	175	92
relative CV of CD71	**<133%**	177	86
relative MFI of CD71	**>46%**	250	126
relative %CD117$^+$ erythroid cells	**37–212%**	182	122

Reference ranges represent values relative to median values for the analyzed markers in the erythroid compartment of normal bone marrow subjects. These values represent 10th and/or 90th percentiles as determined in the set of pathological controls (PC) within the learning cohort. [a]number of PC cases that were available to calculate cut-off values (10th and 90th percentiles); [b]number of NBM cases that were available to calculate median values.
Abbreviations: CV: coefficient of variation; MFI: mean fluorescence intensity.

Correlation between erythroid markers and age

The incidence of MDS increases with age; hence, erythroid markers that are significantly correlated with age may be less suitable for discriminating between MDS and controls. Since we observed significant differences between the groups regarding age (**Table 1**); correlations between FC results for erythroid markers and age were evaluated for normal bone marrow samples. Only CD105 MFI, a variable that was not included in the multivariate analysis, demonstrated a moderate-to-good correlation with age (Spearman's Rho -0.55, p<0.001, n=47, **Supplementary Table 4** and **Supplementary Figure 3**).

Validation of FC aberrancies in the erythroid lineage in MDS and pathological controls

The value of the FC erythroid score was tested in an independent cohort. Nine centers provided data for this validation cohort; the results are depicted in **Figure 4 and Supplementary Table 1**. Similar to results in the learning cohort the relative CVs of CD36 and CD71 were significantly increased in MDS as compared to controls;

CD36 MFI was significantly decreased. Since the distribution of subcategories was similar in the MDS learning and validation cohorts, we compared FC results between the two MDS cohorts. This revealed that the increase in the CD71 CV and the decrease in CD71 MFI were significantly less evident in the MDS validation cohort than in the learning cohort (t-test, p<0.001). Results for CD36 CV and the percentage of CD117$^+$ erythroid cells did not differ between both MDS cohorts (p=0.134 and 0.116, respectively).

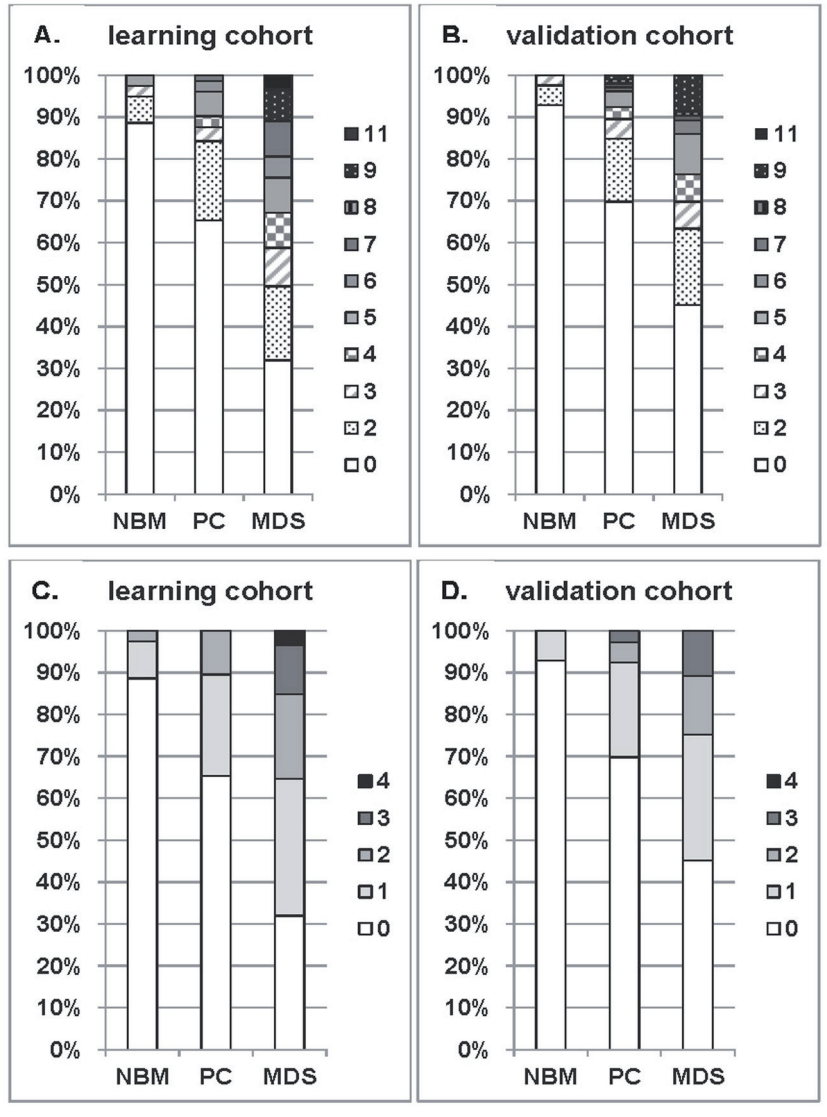


Figure 3 FC-erythroid dysplasia score in the learning and validation cohorts.
The weighted score consists of four parameters, i.e.: increase in CD36 CV (4 points) and CD71 CV (3 points); decrease in CD71 MFI (2 points); and decrease or increase of CD117⁺ erythroid progenitors (2 points). A maximum score of 11 points can be reached. Data are grouped as normal bone marrow (NBM), pathological controls (PC) and MDS (excluding RAEB-1 and -2), relative distribution of the results for the score is displayed along the Y-axes. Panel A. represents the learning cohort consisting of 79 normal bone marrow samples (NBM), 153 pathological controls (PC) and 119 MDS cases. The FC-erythroid dysplasia score could only be calculated in those cases with data on all four defined parameters (351/670 cases). Panel B. represents the results in the validation cohort consisting of 42 NBM samples, 106 pathological controls and 93 MDS cases (241/320 cases; RAEB excluded). Clonal disorders as aplastic anemia and those within the category of essential thrombocythemia, polycythemia vera and primary myelofibrosis were excluded from both cohorts (two and nine cases for learning and validation cohorts, respectively). A cut-off of ≥5 points resulted in a specificity of 90% (95% CI: 84–94%) and a sensitivity of 33% (95% CI: 24–42) in the learning cohort; in the validation cohort, specificity was 92% (95% CI: 86–97%) and sensitivity 24% (95% CI: 15–34%). The numerical score, depicted in panels C and D, consists of four parameters, i.e.: increase in CD36 CV and CD71 CV; decrease in CD71 MFI; and decrease or increase of CD117⁺ erythroid progenitors. A maximum score of 4 points can be reached. A cut-off of ≥2 points resulted in a specificity of 90% (95% CI: 84–94%) and a sensitivity of 35% (95% CI: 27–45) in the learning cohort; in the validation cohort, specificity and sensitivity were 92% (95% CI: 86–97%) and 25% (95% CI: 16–35%), respectively.

Reference ranges as defined in the learning cohort were applied to evaluate the data from the validation cohort and next, to calculate the weighted FC-erythroid dysplasia score. This revealed a specificity of 92% (95% CI: 86–97%) and a sensitivity of 24% (95% CI: 15–34%) for identifying MDS-associated erythroid aberrancies (**Figure 3B and Supplementary Table 5**). In most cases, the numerical score could have been applied (cut-off ≥2 aberrancies; **Figure 3D**); only one MDS had decreased CD71 MFI in combination with an altered CD117⁺ percentage.
To verify the selection of markers that identified FC changes in the erythroid lineage associated with MDS, we performed multivariate logistic regression analysis in the validation cohort and in the combined cohorts. This confirmed the discriminatory power of the CV of CD36 and CD71 and the percentage of CD117⁺ erythroid progenitors, whereas CD71 MFI was replaced by CD36 MFI (**Table 4B–C**). CD105 data were too scarce to be taken into account in the separate cohorts; yet, entering all available markers present in the combined cohorts, multivariate logistic regression analysis identified the combination of CD36 CV, CD71 CV, CD71 MFI and the percentage of erythroid progenitors defined as CD105⁺ instead of CD117⁺ (**Table 4D**).

Figure 4 Distribution of erythroid markers analyzed by flow cytometry among MDS patients and controls within the validation cohort. Results of the analysis of selected markers of the erythroid lineage in the validation cohort are plotted along the X-axes: relative coefficient of variation of CD36 and CD71, and relative percentages of CD117⁺ erythroid progenitors. Normalization was performed against results for the normal bone marrow (NBM) samples of the validation cohort per each individual center. Relative frequencies are depicted along the Y-axes. Dotted lines represent results for NBM samples, dashed lines pathological controls (PC) and solid lines MDS cases. Grey boxes indicate 10th and/or 90th percentiles of pathological controls defined in the learning cohort that were applied for evaluating aberrancies. Note that MDS-RAEB cases were excluded from the MDS group.

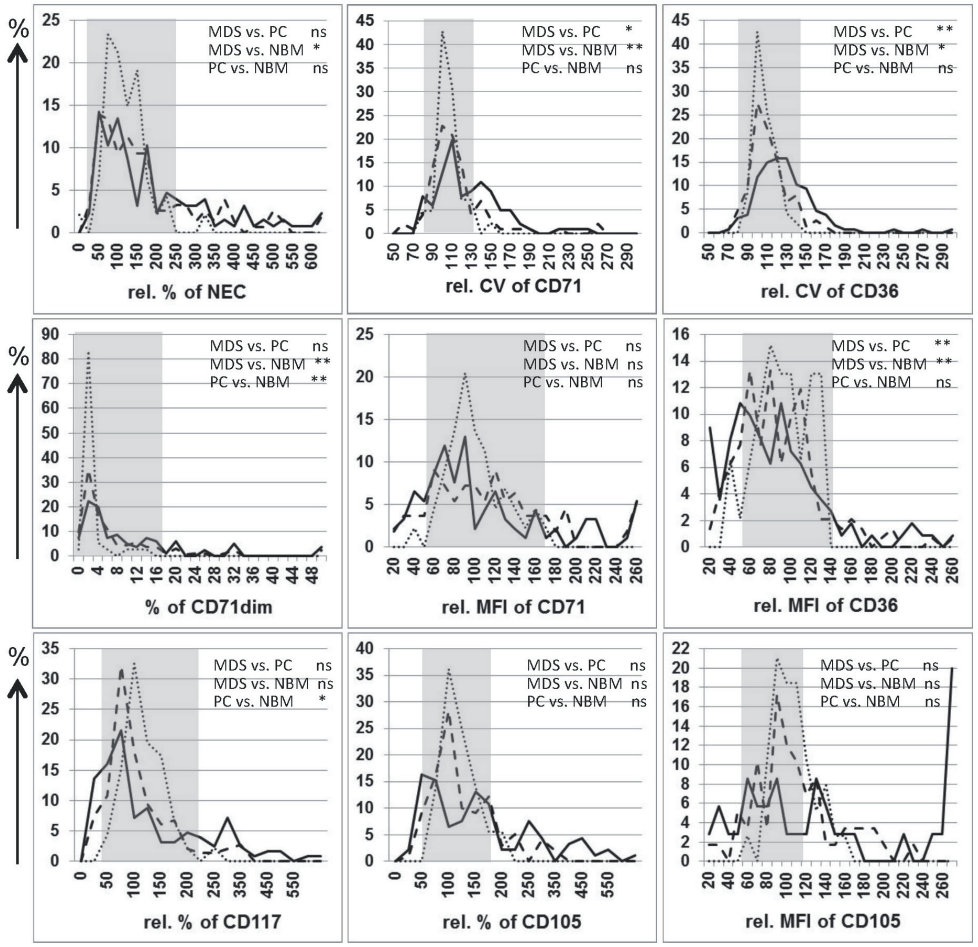


Table 6 Results of FC aberrancies in the erythroid lineage and the FC-erythroid dysplasia score among MDS cases and controls within the learning cohort.

		Increased CV of CD36	Increased CV of CD71	Decreased relative MFI CD71	De/increased %of CD117+ erythroid progenitors		FC-erythroid dysplasia score ≥5	# of cases in flow score[a]
Subcategories								
normal		0	5	4	8		**3**	2/79
pathological control		10	10	10	19		**10**	15/153
MDS[b]		30	46	28	34		**33**	39/119
MDS subcategories								
RCUD		25	31	26	41		**33**	5/15
RARS(-t)		60	64	33	31		**57**	8/14
RCMD		28	44	25	32		**30**	24/79
del(5q)		10	30	38	63		**13**	1/8
RAEB-1/2				40	25			
MDS NOS			80	40				
Pathological control subcategories								
iron deficiency anemia		11	0	0	11		**6**	1/18
iron incorporation disturbances or anemia in chronic disease		7	10	3	23		**10**	3/29
vitamin B12/folic acid deficiencies		0	17	0	25		**0**	0/6
anemia in auto-immune diseases		0	0	20	0		**0**	0/5
anemia due to renal failure		25	0	0	60			
anemia other[c]		0	22	11	0		**0**	0/5
cytopenia associated with marrow infiltration		0	20	16	40			
cytopenia induced by chemotherapy or medication or post-SCT		10	0	24	9		**0**	0/7
ITP or neutropenia or auto immune cytopenia NOS		17	9	12	9		**14**	3/21
reactive conditions or cytopenia induced by infections		19	39	24	19		**28**	5/18
normal bone marrow (peripheral cytopenia NOS)		0	20	33	67		**20**	1/5
other than defined subcategories NOS		5	0	4	16		**0**	0/20
inconclusive		0	0	0	18		**0**	0/11

Displayed numbers correspond to the percentage of cases per subgroup that were beyond the reference ranges (Table 5). The CV for CD71 and CD36 were tested against the 90th percentile; the expression level (MFI) of CD71 was against the 10th percentile; and the percentage of CD117 erythroid progenitor cells was tested against both the 10th and 90th percentiles. Only data of subsets with five or more cases are depicted. Diagonally marked cells represent data not available or reliable (i.e., missing or only small data sets (<5 cases)) Note: [a]number of cases with a FC-erythroid dysplasia score of ≥2 per total number of cases in which all parameters were available that comprise the score; [b]MDS subcategory RAEB is excluded from these results; [c] the subcategory "anemia other" comprises among others cases of normocytic anemia, anemia unexplained; NOS: not otherwise specified.

Discussion

Analysis of erythroid dysplasia is rarely included in current FC protocols for MDS, since the significance of FC data from the erythroid lineage is, to a large extent, still under debate.[3] Here, we report the results of a multicenter study within the IMDSFlow group, which focused on defining erythroid parameters that enable discrimination of dyserythropoiesis associated with MDS from non-clonal cytopenia. The results revealed that aberrancies in the erythroid markers CD71 and CD36 (expressed as the CV), together with the MFI of CD71 and an abnormal percentage of CD117^{+} erythroid progenitor cells within the distinct stages of erythroid differentiation provided the best discrimination between MDS and non-clonal cytopenia. A weighted score was based on these four parameters; this resulted in a specificity of 90% (95% CI: 84–94%) and a sensitivity of 33% (95% CI: 24–42) in the learning cohort; in the validation cohort, specificity was 92% (95% CI: 86–97%) and sensitivity 24% (95% CI: 15–34%). Specificity of the defined markers for identification of MDS-associated erythroid changes by FC is considered to be more important than its general diagnostic value for MDS. Hence, increasing the cut-off from ≥5 to ≥6 points would optimize the specificity (96% in both cohorts); however, at the cost of a decrease in sensitivity (24% and 14%, in the learning and validation cohort, respectively). The lower sensitivity compared to the learning cohort could be explained by a less evidently increased CD71 CV and decreased CD71 MFI in the MDS validation cohort. Hence, fewer MDS cases scored CD71 CV and/or CD71 MFI as aberrant (**Supplementary Table 3)**.

A numerical score based on one point per aberrancy and a cut-off of ≥2 aberrant markers led to similar results. Yet, it must be taken into account that the sole combination of CD71 MFI and percentage CD117^{+} erythroid progenitors is not sufficient to indicate MDS-associated changes in the erythroid lineage by FC. Ultimately, the analysis of the erythroid and myelomonocytic lineages and hematogones should be combined. Future validation should reveal the power of the herein defined erythroid markers. In addition, it is relevant to elucidate the value of the combination of myeloid and erythroid FC markers in indeterminate cases according to cytomorphology.

The multicentric data presented herein confirmed results from a recent study that reported a significant increase in the CV of CD71 and CD36 expression highly suggestive

for MDS.[27] Yet, discrimination between MDS and controls based on CV values was less clear for the present dataset. It was stated that the difference in CD71 CV between MDS and controls was less pronounced after erythrocyte lysis; however, this was not the case for CD36 CV.[27] All data in the present study were obtained after erythrocyte lysis, which might explain the observed differences. It may seem paradoxical to use erythrocyte lysing solutions when the focus is on analysis of the erythroid lineage. Lyse-stain-wash is the recommended protocol for processing samples for FC in MDS.[2] Despite IMDSFlow recommendations, methodological variation between centers may have attributed to differences in results as demonstrated in normal controls. Harmonization or even standardization of methods may narrow differences and improve validity of conclusions from multicenter studies as has been demonstrated with respect to lymphocyte screening within the Euroflow consortium.[28] Hence, grouping of data per technical procedure could have been informative from a practical perspective; yet, the power of the analyses within and between numerous subgroups of centers would have would have been strongly limited by sample sizes. Notably, in daily practice, FC results in subjects suspected for MDS should always be compared with a center's own cohort of control samples.

Despite technical considerations, our data confirm the robustness of the evaluation of an increase in the CD71 and CD36 CV on erythroid cells.[27] Another solid marker in the multivariate analyses in the herein presented cohorts was the evaluation of the percentage of erythroid progenitors defined as CD117$^+$. A potential marker for future inclusion in erythroid data analysis by FC is CD105. In the multivariate logistic regression analysis of the combined cohorts, the evaluation of the percentage of CD117$^+$ erythroid progenitors was replaced by the percentage CD105$^+$ erythroid progenitors, though, these results were based on data from only 6/19 participating centers. Application of CD105 may overcome the potential error of selection of CD117$^+$ erythroid progenitors without the aid of other markers such as a myeloid marker. Moreover, it has been demonstrated (in normal and pathological controls) that CD105 is lost before carbonic anhydrase is expressed, which suggests that the majority of CD105$^+$ cells are not subject to ammonium chloride-based lysing protocols.[15]

Next to that, CD105 overexpression was confirmed in some cases of MDS in our dataset.[16, 18] However, we also observed a decreased expression in MDS; notably CD105 expression was negatively correlated with age in normal controls. Future studies in larger data sets may elucidate if CD105 is truly valuable in analysis of erythroid dysplasia in MDS.

Cytomorphology reports dysplastic features in erythropoiesis in non-MDS cases, such as reactive conditions.[21-23] Moreover, patients with cytopenia due to marrow

infiltration may demonstrate FC aberrancies associated with MDS. MDS may even coincide with the other malignancy in these patients.[29, 30] A subset of patients with reactive marrows or marrow infiltration in our dataset indeed showed multiple erythroid aberrancies (5/23 and 2/9, respectively; **Supplementary Table 5**). Follow-up analysis after several months may demonstrate MDS in these cases.[31] This stresses that FC analysis in suspected MDS, though proven very specific, should always be part of an integrated diagnostic approach rather than a solitary diagnostic tool.[32]

New insights may improve the impact of FC in the diagnosis of MDS. A recent report showed that increased expression of CD44 on all maturational stages of erythroid cells was associated with MDS, irrespective of presence or absence of morphologic dyserythropoiesis.[33] In addition, decreased expression of the major coxsackie adenovirus receptor (CAR) was demonstrated in dysplastic CD105$^+$ erythroid progenitors.[34, 35]

To summarize, we identified significant aberrancies with respect to the FC markers recommended by IMDSFlow for analysis of the erythroid lineage in MDS. The best indicators of dysplastic changes associated with MDS were an increased CV of CD36 and CD71, a decreased MFI of CD71 in combination with decreased or increased percentages of erythroid progenitors (CD117$^+$). The defined FC-erythroid dysplasia score demonstrated high specificity. Future studies should assess the contribution of the selected erythroid markers to the evaluation of the myeloid progenitors, the maturing myelomonocytic lineage and hematogones in current FC protocols in MDS. This will be implemented in an upcoming multicenter data collection exercise within IMDSFlow.

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Supplementary files

Table S1 Comparison of results of the FC analysis of the erythroid lineage stratified by cohort.

	p-value MDS vs. PC	p-value MDS vs. NBM	p-value PC vs. NBM
Learning cohort			
Relative %NEC	<0.001	<0.001	0.503
Pattern CD71/CD235a	<0.001	<0.001	<0.001
%CD71dim	<0.001	<0.001	0.296
Relative MFI of CD71	<0.001	<0.001	0.619
Relative CV of CD71	<0.001	<0.001	0.485
Relative MFI of CD36	0.001	<0.001	0.139
Relative CV of CD36	<0.001	<0.001	0.020
Relative %CD117 progenitors	0.711	0.787	0.536
Relative %CD105 progenitors	0.399	0.179	0.355
Relative MFI of CD105	0.136	0.002	0.008
Validation cohort			
Relative %NEC	0.368	0.022	0.106
Pattern CD71/CD235a	<0.001	<0.001	0.284
%CD71dim	0.092	<0.001	<0.001
Relative MFI of CD71	0.180	0.284	0.198
Relative CV of CD71	0.002	<0.001	0.639
Relative MFI of CD36	0.145	0.014	0.101
Relative CV of CD36	<0.001	<0.001	0.254
Relative %CD117 progenitors	0.910	0.148	0.010
Relative %CD105 progenitors	0.162	0.217	0.805
Relative MFI of CD105	0.304	0.168	0.715

Note: Collected data were normalized against the median value determined in the set of normal bone marrow samples, except for aberrant pattern of CD71 vs. CD235a and %CD71dim. P-values represent the results of the comparison performed by the Kruskal Wallis test, except for aberrant pattern of CD71 vs. CD235a which was analyzed by the Fisher's exact test. Abbreviations: dim: diminished; CV: coefficient of variation; MDS: myelodysplastic syndromes; NBM: normal bone marrow samples; NEC: nucleated erythroid cells; PC: pathological controls. MDS-RAEB cases were excluded from this analysis.

Table S2 Cut-offs used for evaluation of results from the FC analysis of erythroid markers in the learning cohort.

	10th percentile	90th percentile	# of PC cases[a]	# of NBM cases
Relative %NEC	-	268%	238	139
%CD71dim	-	17%	250	129
Relative MFI of CD71	45%	-	250	126
Relative CV of CD71	-	133%	165	88
Relative MFI of CD36	53%	-	203	124
Relative CV of CD36	-	145%	177	92
Relative %CD117 progenitors	37%	222%	180	122
Relative %CD105 progenitors	50%	184%	52	59
Relative MFI of CD105	52%	113%	70	47

Note: cut-off values represent the 10th and 90th percentiles of results for erythroid markers among pathological controls in the learning cohort. The number of pathological control (PC) cases that were available to calculate cut-off values are displayed (a). Most values (except for %CD71dim) are expressed as ratio to the median value determined in the set of normal bone marrow samples. The utmost right column displays the number of normal bone marrow (NBM) cases that were available to calculate these median values. Abbreviations: CV: coefficient of variation; dim: diminished; MFI: mean fluorescence intensity; NEC: nucleated erythroid cells.

Example on how to translate these reference ranges for application in a single center: If sufficient data are available on a set of pathological controls, 10th and 90th percentiles can be determined. Otherwise, collect data on an appropriate amount of normal bone marrow samples (min. 10) and determine median values for the parameters in the erythroid score. For instance, the median CV value of CD71 in the normal bone marrow samples is 67 and the median percentage of CD117+ erythroid progenitors in the erythroid compartment is 8%. Then the reference values are: CD71 CV: 133/100*67=89; a CD71CV>89 should be considered increased. Similarly for %CD117+: lower cut-off 37/100*8=3.0 and highest cut-off 222/100*8=17.8; i.e. a CD117+ percentage (erythroid compartment) below 3.0% or above 17.8% should be considered aberrant. Otherwise, in case a sufficient amount of data is present regarding a large variation of pathological controls, 10th and 90th percentiles calculated from a center's own cohort may be applied when comparable to the herein described reference values.

Table S3 Percentage of flow cytometric aberrancies in the erythroid lineage among MDS and controls.

	NBM	PC	MDS
Learning cohort			
Relative %NEC	2.9	10.1	23.1
Pattern CD71/CD235a	3.7	17.3	64.9
%CD71dim	0.8	10.0	31.5
Relative MFI of CD71	4.0	10.0	27.8
Relative CV of CD71	4.5	10.3	45.5
Relative MFI of CD36	0.8	10.3	25.8
Relative CV of CD36	0.0	10.2	30.1
Relative %CD117 progenitors (decreased) (increased)	8.2 (4.9) (3.3)	19.4 (10.0) (9.4)	33.8 (15.2) (18.6)
Relative %CD105 progenitors (decreased) (increased)	6.7 (3.3) (3.3)	20.8 (7.8) (13.0)	48.4 (26.6) (21.9)
Relative MFI of CD105 (decreased) (increased)	29.8 (4.3) (25.5)	22.5 (11.3) (11.3)	58.7 (38.1) (20.6)
Validation cohort			
Relative %NEC	2.1	18.7	23.0
Pattern CD71/CD235a	4.8	16.7	54.5
%CD71dim	4.8	20.6	19.8
Relative MFI of CD71	2.3	8.9	10.6
Relative CV of CD71	2.1	16.7	27.3
Relative MFI of CD36	6.5	14.6	27.4
Relative CV of CD36	0.0	5.3	19.7
Relative %CD117 progenitors (decreased) (increased)	2.2 (0.0) (2.2)	20.1 (13.4) (6.7)	40.8 (22.4) (18.4)
Relative %CD105 progenitors (decreased) (increased)	2.8 (0.0) (2.2)	24.2 (10.1) (14.1)	45.7 (18.5) (27.2)
Relative MFI of CD105 (decreased) (increased)	23.7 (0.0) (23.7)	47.0 (7.6) (39.4)	55.8 (7.8) (48.1)

Numbers represent percentages of cases that scored abnormal when compared with the defined cut-offs for a particular marker (Supplementary Table 2.). Abbreviations: dim: diminished; CV: coefficient of variation; MDS: myelodysplastic syndromes; NBM: normal bone marrow samples; NEC: nucleated erythroid cells; PC: pathological controls. MDS-RAEB cases were excluded from this analysis.

Table S4 Correlation of age and FC markers of the erythroid lineage in normal bone marrow samples within the learning cohort

	Spearman's Rho	p-value
Relative %NEC	0.12	0.179
Pattern CD71/CD235a	0.08	0.366
%CD71dim	0.13	0.153
Relative MFI of CD71	-0.20	0.032
Relative CV of CD71	0.17	0.148
Relative MFI of CD36	0.21	0.026
Relative CV of CD36	-0.01	0.925
Relative %CD117 progenitors	-0.05	0.640
Relative %CD105 progenitors	0.30	0.136
Relative MFI of CD105	-0.55	**<0.001**

Abbreviations: CV: coefficient of variation; MFI: mean fluoresce intensity; NEC: nucleated erythroid cells

Table S5 Results of the FC erythroid dysplasia score among MDS cases and controls stratified per cohort.

	Learning Cohort		Validation Cohort		Combined Cohorts	
	FC erythroid dysplasia score ≥5	# of cases	FC erythroid dysplasia score ≥5	# of cases	FC erythroid dysplasia score ≥5	# of cases
Subset						
normal	3	2/79	0	0/42	2	2/121
pathological controls	10	15/153	8	8/106	9	23/259
MDS[a]	33	39/119	24	22/93	29	61/212
MDS subcategories						
RCUD	33	5/15	43	3/7	41	9/22
RARS(-t)	57	8/14	43	3/7	52	11/21
RCMD	30	24/79	22	12/58	26	36/137
del(5q)	13	1/8	33	1/3	27	3/11
RAEB-1/2			15	2/13	27	4/15
MDS not specified			6	1/16	6	1/16
Pathological control subcategories						
iron deficiency anemia	6	1/18	9	1/11	7	2/29
iron incorporation disturbances or anemia in chronic disease	10	3/29	20	1/5	12	4/34
vitamin B12/folic acid deficiencies	0	0/6	14	1/7	8	1/13
anemia in auto-immune diseases	0	0/5			17	1/6
anemia due to renal failure		1/3		0/2	20	1/5
anemia other[b]	0	0/5	20	1/5	10	1/10
cytopenia associated with marrow infiltration		1/3	17	1/6	22	2/9
cytopenia induced by chemotherapy or medication or post-SCT	0	0/7	0	0/6	0	0/13
ITP or neutropenia or auto immune cytopenia NOS	14	3/21	0	0/6	15	3/27
reactive conditions or cytopenia induced by infections	28	5/18	0	0/5	22	5/23
normal bone marrow (peripheral cytopenia NOS)	20	1/5			20	1/5
other subcategories	0	0/20	0	0/22	0	0/42
inconclusive	0	0/11	0	0/7	0	0/18
non clonal cytopenia NOS			0	0/18	0	0/18

Note: Data represent percentage of subjects with an FC erythroid score of ≥2 and the actual amount of 'positive' cases per available cases. Only data of subsets with five or more cases are depicted; diagonally marked cells represent data not available or reliable (i.e., missing or only small data sets (<5 cases)). aMDS subcategory RAEB is excluded from these results; bthe subset "anemia other" contains among others cases of normocytic anemia, anemia unexplained, etc.; NOS: not otherwise specified.

Figure S1 Results for erythroid markers in normal bone marrow samples analyzed by FC within different centers (learning cohort). Data are presented as boxplots with median and range. Depicted erythroid markers are percentage of nucleated erythroid cells (NEC), relative (rel.) expression of CD71, rel. CV of CD71 expression, rel. expression of CD36, rel. CV of CD36 expression, subset of CD71dimCD235a+ cells in CD71-CD235a pattern, rel. expression of CD105, percentage of CD117+ cells in the erythroid compartment and percentage of CD105+ cells in the erythroid compartment. The different centers were anonymized (A to S) in order of data entry in the cohort. Open boxes reflect application of ammonium chloride as lysing procedure (PharmLyse, BD Biosciences or home-made; yellow boxes: Versalyse (BeckmanCoulter); green boxes FACSLyse (BD Biosciences); blue-edged boxes indicate lysis at 4ºC, red-edged boxes at 37ºC and black boxes at room temperature. Centers M and R use longest duration of incubation with lysing solution 20 and 25 minutes, respectively. No data for normal bone marrow samples were are available for centers H and O; center S only entered data in the validation cohort. Asterisks indicate the procedures in these centers; red: ammonium chloride at 37ºC; black: ammonium chloride at room temperature; green: FACS lyse at room temperature. Abbreviations: LSW: lyse-stain-wash; SLW: stain-lyse-wash. Centers F and J applied LSW in a tube containing CD235a and SLW in other tubes.

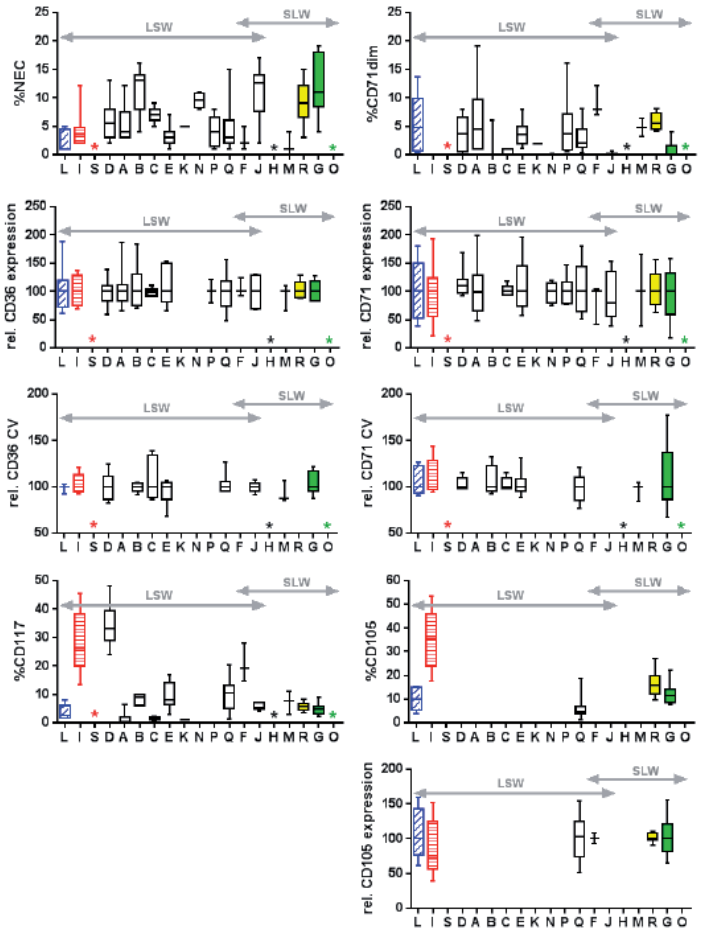


Figure S2 Results for erythroid markers in normal bone marrow samples analyzed by FC within different centers (learning cohort). Data are presented as boxplots with median and range. Depicted erythroid markers are percentage of nucleated erythroid cells (NEC) and the percentage of CD117+ cells in the erythroid compartment before and after normalization (upper and lower panel, respectively). The different centers were anonymized (A to S) in order of data entry in the cohort. Open boxes reflect application of ammonium chloride as lysing procedure (PharmLyse, BD Biosciences or home-made; yellow boxes: Versalyse (BeckmanCoulter); green boxes FACSLyse (BD Biosciences); blue-edged boxes indicate lysis at 4ºC, red-edged boxes at 37ºC and black boxes at room temperature. Centers M and R use longest duration of incubation with lysing solution 20 and 25 minutes, respectively. No data for normal bone marrow samples were are available for centers H and O; center S only entered data in the validation cohort. Asterisks indicate the procedures in these centers; red: ammonium chloride at 37ºC; black: ammonium chloride at room temperature; green: FACS lyse at room temperature. Abbreviations: LSW: lyse-stain-wash; SLW: stain-lyse-wash. Centers F and J applied LSW in a tube containing CD235a and SLW in other tubes

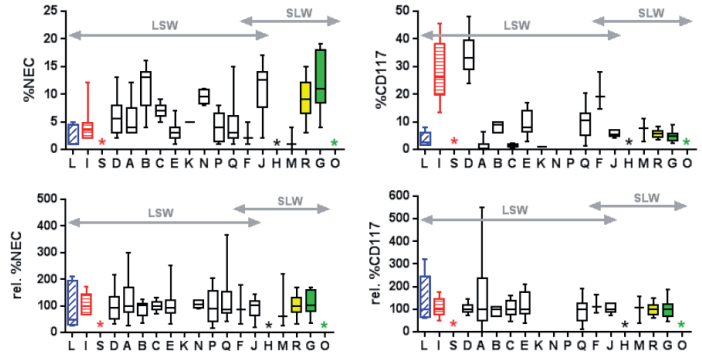
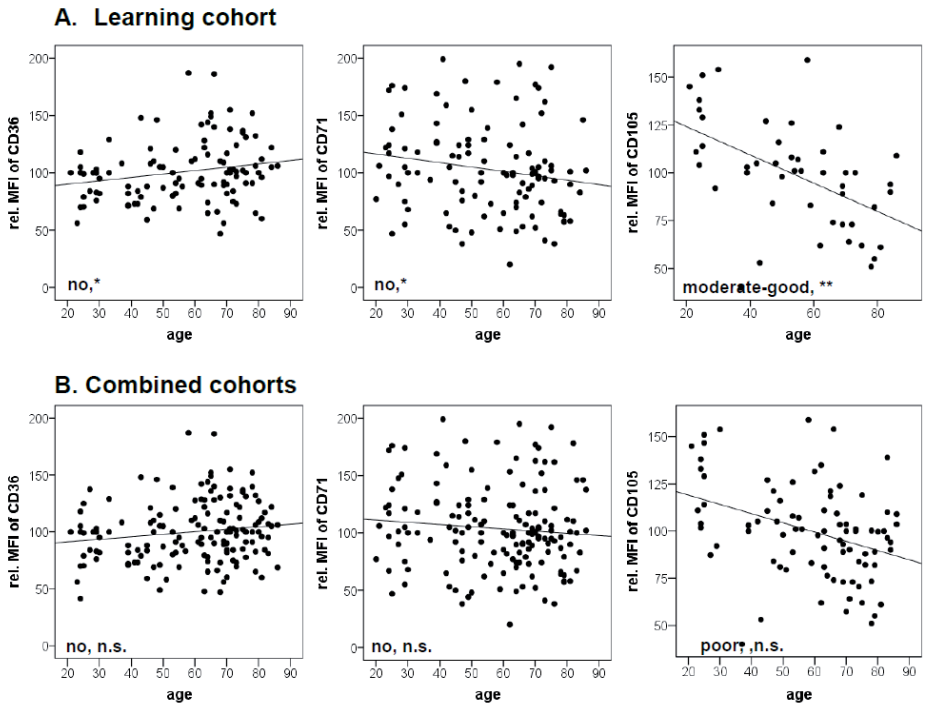


Figure S3 Correlation of age and FC markers of the erythroid lineage in normal bone marrow samples within the learning cohort and the combination of learning and validation cohorts. Scatterplots are displayed for markers that showed a significant relation with age in the learning cohort (panel A): relative MFI of CD36, CD71 and CD105. The results of the same markers in the combined learning and validation cohorts are displayed in Panel B. Spearman's Rho and p-values are indicated in the plots as no: Spearman's Rho -0.2–0.2; poor: Spearman's Rho -0.2– -0.5; moderate-to good: Spearman's Rho -0.5– -0.7; and n.s.: p>0.05; *: p<0.05; **: p<0.001, respectively.



CHAPTER 5

IMPLEMENTATION OF ERYTHROID LINEAGE ANALYSIS BY FLOW CYTOMETRY IN DIAGNOSTIC MODELS FOR MYELODYSPLASTIC SYNDROMES

A STUDY ON BEHALF OF THE HOVON89 STUDY GROUP

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HAEMATOLOGICA. 2017; 102:320-326.

Abstract

Flow cytometric analysis is a recommended tool in the diagnosis of myelodysplastic syndromes. Current flow cytometric approaches evaluate the (im)mature myelo-/monocytic lineage with a median sensitivity and specificity of ~71% and ~93%. We hypothesized that addition of erythroid lineage analysis could increase the sensitivity of flow cytometry. Hereto, we validated the analysis of erythroid lineage parameters recommended by the International/European LeukemiaNet Working Group for Flow Cytometry in Myelodysplastic Syndromes and incorporated this evaluation in currently applied flow cytometric models. One hundred and sixty-seven bone marrow aspirates were analyzed, 106 patients with myelodysplastic syndromes and 61 cytopenic controls. There was a strong correlation between presence of erythroid aberrancies assessed by flow cytometry and the diagnosis myelodysplastic syndromes, validating the previously described erythroid evaluation. Furthermore, addition of erythroid aberrancies to two different flow cytometric models led to an increased sensitivity to detect myelodysplastic syndromes: from 74% to 86% for the addition to the diagnostic score designed by Ogata and colleagues, and from 69% to 80% for the addition to the integrated flow cytometric score for myelodysplastic syndromes, designed by our group. In both models the specificity was unaffected. The high sensitivity and specificity of flow cytometry in the detection of myelodysplastic syndromes illustrates the important value of flow cytometry in a standardized diagnostic approach. The trial is registered at www.trialregister.nl as NTR1825; EudraCT nr.:2008-002195-10

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic disorders characterized by cytopenia(s) and risk of leukemic transformation.[1] Multi-parameter flow cytometric (FC) analysis is a recommended tool to support the diagnosis of MDS, which is based on dysplastic features by cytomorphology and typical cytogenetic abnormalities.[2] The International/European LeukemiaNet Working Group for Flow Cytometry in MDS (IMDS-flow) provided recommendations on how to process and analyze bone marrow aspirates of patients with unexplained cytopenias suspected of MDS.[3,4] Analytic methods have been developed and validated for characterization and quantification of dysplasia and enable accurate diagnosis of MDS.[5–12] The most straightforward is a four-parametric diagnostic score that integrates: percentage of CD34-positive myeloid progenitors, percentage of B cell progenitors within the CD34-positive compartment, CD45 expression level of CD34-positive myeloid progenitors (related to CD45 expression level on lymphocytes), and sideward light scatter peak channel value (SSC) of granulocytic cells (related to SSC of lymphocytes). This diagnostic score has a sensitivity and specificity of 69% and 92%, respectively in low-intermediate risk MDS.[13,14] More elaborate scores can reach specificities up to 100%; this however is accompanied by lower sensitivities.[15] In accordance with recommendations issued by the IMDS-flow, our group designed and validated an integrated MDS-FC score (iFS).[16] The iFS comprises the diagnostic score and evaluation of frequently described aberrant expression levels of lineage defining markers and presence of lineage infidelity markers on (im)mature myelo-/monocytic cells. Sensitivity and specificity of the iFS within a large cohort of patients with persistent cytopenias of unknown origin, were 63% and 98%, respectively.[17] The lower sensitivity in this and other reports can be explained by the fact that most MDS-FC approaches only evaluate the myeloid cell compartment. Since dyserythropoiesis is the most prevalent feature by cytomorphology in MDS, addition of in depth evaluation of the erythroid compartment is expected to improve sensitivity.[18] MDS patients with erythroid dysplasia but without dysmyelopoiesis, may then be identified by FC.

For evaluation of the erythroid compartment different antibody combinations of CD45, CD235a, CD71, CD36, CD105, and intracellular markers such as cytosolic H-ferritin, cytosolic L-ferritin and mitochondrial ferritin have been described.[19–22] The IMDS-flow group recently proposed guidelines for erythroid evaluation advising the evaluation of CD36 coefficient of variation (CV), CD71 CV and mean fluorescence intensity (MFI), and percentage of progenitors (CD117 positive within CD45 negative-diminished cell fraction) within the erythroid compartment. Sensitivity and specificity of this marker combination for the detection of MDS-associated erythroid aberrancies were 35% and 90%, respectively. The current study aimed to validate these erythroid parameters in an independent cohort of patients diagnosed with MDS treated within a prospective

clinical study and in a reference group of patients with proven non-clonal cytopenias. Furthermore, the additive value of erythroid evaluation to currently applied MDS-FC diagnostic approaches was explored.

Table 1 Erythroid markers that comprise the IMDS-Flow erythroid FC score and the cumulative score, stratified by patient group.

	CV CD71	%	MFI CD71	%	CV CD36	%	% CD117	%	≥2 points	%
Control group	**14/61**	**23**	**3/61**	**5**	**2/61**	**3**	**35/61**	**57**	**7/61**	**11**
Alcohol abuse	0/2	0	1/2	50	0/2	0	0/2	0	0/2	0
Aplastic anemia	2/3	67	0/3	0	0/3	0	0/3	0	0/3	0
Auto-immune cytopenia	3/10	30	0/10	0	1/10	10	8/10	80	3/10	30
Chronic disease	0/3	0	0/3	0	0/3	0	3/3	100	0/3	0
*Eosinophilia**	0/1	0	0/1	0	0/1	0	1/1	100	0/1	0
Iron deficiency	6/26	23	1/26	4	1/26	4	15/26	58	4/26	15
Iron incorporation disorder	2/10	20	0/10	0	0/10	0	4/10	40	0/10	0
Medication caused cytopenia	0/3	0	0/3	0	0/3	0	3/3	100	0/3	0
Vitamin B12 deficiency	1/3	33	1/3	33	0/3	0	1/3	33	0/3	0
MDS group	**70/106**	**66**	**22/106**	**21**	**36/106**	**34**	**64/106**	**60**	**68/106**	**64**
RCUD	2/4	50	0/4	0	2/4	50	3/4	75	3/4	75
RARS	19/20	95	6/20	30	9/20	45	8/20	40	17/20	85
RCMD	12/23	52	4/23	17	8/23	35	15/23	65	13/23	57
RCMD-RS	19/27	70	7/27	26	8/27	30	20/27	74	19/27	70
RAEB-1	11/14	79	2/14	14	7/14	50	6/14	43	10/14	71
Isolated del(5q)	3/12	25	1/12	8	2/12	17	10/12	83	4/12	33
MDS-U	1/2	50	0/2	0	0/2	0	1/21/4	50	0/2	0
CMML	3/4	75	2/4	50	0/4	0		25	2/4	50

*Normal bone marrow by cytomorphological assessment. The CD36 CV is the most specific parameter (2/61 control patients; 3%), and CD71 CV is the most sensitive parameter (70/106 MDS patients; 66%). In summary: 11% of the controls and 64% of the MDS patients show MDS-associated erythroid aberrancies, as defined by ≥2 points.

Methods

Patients

A well-defined MDS group and cytopenic control group were assembled between May 2009 and July 2014 (**Table 1**). The MDS group consisted of patients enrolled in the HOVON89 study (trial registered at www.trialregister.nl as NTR1825; EudraCT nr.: 2008-002195-10). Bone marrow aspirates for FC analysis were taken prior to inclusion, MDS was diagnosed in accordance with the minimum diagnostic criteria established by the WHO 2001 criteria.[23] The definition of non-clonal cytopenias was based on clinical characteristics, cytomorphology, cytogenetic and biochemical indicators. The median age of the MDS group was 71 (range 38-85) median age of the control group was 65 (range 23-91). The research program was approved by the local ethics committee, and all patient-related research strictly abided by the Declaration of Helsinki.

Sample Preparation, Antibody Combinations, and Cell Acquisition

Sample processing was performed according to ELN guidelines for FC within 24 hours.[15] A 4-color analysis was performed from 2009-2012, and an 8-color analysis from 2012-2014. The staining panels are outlined in the supplementary file (**Table S1**). At least 100,000 CD45-positive events were acquired, using a FACSCalibur™ or FACSCantoII™ (BD Biosciences, San Jose, CA, USA). Cells were analyzed using Cell QuestPro (BD Biosciences) or Infinicyt software (Cytognos, Salamanca, Spain), respectively. Gating was performed as previously described.[15,24]

MDS-FC scores

For evaluation of the erythroid compartment, guidelines as described by the IMDS-flow were applied. Erythroid evaluation included analysis of CD71 (CV and MFI), CD36 (CV) and CD117 (percentage within the CD45-negative-diminished cell fraction). Cut-off values were assessed as described in the tandem-paper (see also the mathematical examples in the supplementary files of the paper). Examples are provided in the supplementary file (**Figure S1**). Following the simplified recommendations: an increased CV of CD71, a decreased MFI of CD71, an increased CV of CD36, and a decreased or increased percentage of CD117 were each assigned *one* point. A score of ≥2 points was defined as MDS-associated erythroid aberrancies. The four parameter diagnostic score was calculated according to guidelines as previously described, using the defined cut-offs.[13] The iFS was established as described previously.[25] The diagnostic score, the iFS and the erythroid score are described in **Table 2A**.

Models for incorporation of erythroid analysis

Tables 2B-2C describe the two models designed to add erythroid FC analysis to validated MDS-FC approaches. Patients with MDS-associated erythroid aberrancies received one extra point in comparison with the original diagnostic score, a total of ≥2 points was labeled as MDS. The second model added erythroid evaluation to the iFS. Patients with iFS results B *with* erythroid aberrancies by FC were labeled compatible with MDS.

Statistics

Results from MDS-FC were compared between the MDS and control group. Absolute numbers and relative percentages described the data. To test the concordance between presence of MDS-associated erythroid aberrancies and patient group a chi-square test was performed. To compare the results of different techniques the McNemar test was used. P-values <0.05 were statistically significant. Specificity and sensitivity and 95%-confidence intervals were calculated for each MDS-FC model using a two-by-two model. Inter-observer analysis of MDS-FC aberrancies and the diagnostic score was tested by an independent MDS-expert center, the Department of Immunology of the Erasmus University Medical Center, Rotterdam, The Netherlands. Analyses were performed using PASW Statistics version 20.0 (SPSS, Chicago, IL).

Results

Evaluation of erythroid markers

In accordance with the IMDS-flow recommendations we analyzed CD36 (CV), CD71 (CV and MFI), and CD117 (percentage within the CD45 negative-diminished cell fraction). **Table 1** lists the analyzed erythroid markers per group. An increased CV of CD71 was the most *sensitive* marker for MDS as it was positive in 66% of MDS patients, followed by an increased/decreased percentage of CD117 (64%). An increased CV of CD36 was the most *specific* marker as only 3% of controls were positive for this marker. Within the MDS group 64% patients showed multiple erythroid aberrancies (≥2 points), compared to 11% of patients within the control group. The presence of multiple erythroid aberrancies was significantly correlated with the diagnosis of MDS (p<0.001).

Correlation between patient group and cytomorphology

As we found a significant correlation between patient group (MDS or control) and the presence of erythroid aberrancies, the next step was to evaluate the relation between erythroid evaluation by cytomorphology and FC in more detail. As controls might have minimal dyserythropoiesis by cytomorphology, FC might also detect erythroid aberrancies in controls.[26,27] Information about erythroid features by cytomorphology was available in 92% patients in the MDS group and in 98% patients within the control group. **Table 3** provides an overview of the results. Although the positive test results (dyserythropoiesis by cytomorphology and erythroid aberrancies by FC) seem equally distributed between the MDS and the controls, FC identified more dysplastic cases than cytomorphology (MDS-FC-positive cases within the cytopenic controls based on morphology). Therefore the McNemar test which focuses on the differences between two correlated proportions, was not significant (P=0.01).

Table 2A The parameters that describe the original integrated MDS-FC score, the erythroid score and the diagnostic score.

Diagnostic Score	Myeloid progenitors	Granulocytes**	Monocytes**	Erythrocytes
Two of the following: Increased percentage of myeloid progenitor cells Abnormal expression of CD45 on myeloid progenitor cells Decreased SSC on granulocytes Decreased percentage of B cell progenitor cells	>5% myeloid progenitors OR: <5% myeloid progenitors with one of the following: Lymphoid markers present (CD2, CD5, CD19, CD25, CD56) OR: <5% myeloid progenitors with two of the following: Decrease in CD45 expression Abnormal expression of CD34 Abnormal expression of CD117 Abnormal expression of CD13 Abnormal expression of CD33 Abnormal expression of HLA-DR Expression of CD11b Expression of CD15*	Two of the following: Decreased SSC Abnormal CD11b/CD13 Abnormal CD16/CD13 Expression of HLA-DR Lack of CD33 expression Asynchronous shift to the left Abnormal expression of CD15 OR: Presence of lymphoid markers OR: Presence of CD34 on mature myeloid cells OR: Myeloid/Lymphoid ratio < 1	Two of the following: Abnormal CD45/SSC Decreased/increased number as compared to lymphocytes Abnormal CD11b Abnormal HLA-DR Abnormal CD11b/HLA-DR Abnormal expression of CD14 Abnormal expression of CD13 Loss of CD16 Abnormal expression of CD33 OR: Presence of lymphoid markers OR: Presence of CD34 on mature monocytic cells	Two of the following***: Increased CD36 coefficient of variation Increased CD71 coefficient of variation Decreased expression of CD71 Decreased / increased percentage of CD117 positive within nucleated erythroid cells

*Note that normal myeloid progenitors might also express CD15. **The granulocytic and monocytic cell compartments were integrated into one compartment in Table 2C (the iFS). *** in case of aberrant CD71 percentage and CD117 percentage one extra abnormality is mandatory. This figure is adapted from Wells et al, scores adjusted as by Cutler et al, and Cremers et al.[7,17,25]

Table 2B The addition of the erythroid evaluation to the diagnostic score. [13,14]

Diagnostic score	0	0		1	1	
Aberrant erythroid	-	+		-	+	
MDS according to FC	No	No		No	Yes	
Diagnostic score	≥2	≥2		≥2	≥2	
Aberrant erythroid	-	+		-	+	
MDS according to FC	Yes	Yes		Yes	Yes	

Table 2C The addition of the erythroid evaluation to the integrated MDS-FC score (iFS).[16]

Diagnostic score	<2 abnormalities								≥2 abnormalities							
Aberrant myeloid progenitors	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+
Aberrant neutrophils (≥2 other aberrancies)	-	-	+	+	-	-			-	-	+	+	-	-		
Aberrant monocytes (CD56 / ≥2 aberrancies)																
Original iFS*	A	A	A/B	A/B	A/B	A/B	C	C	A/B	A/B	B/C	B/C	B/C	B/C	C	C
Aberrant erythroid (≥2 aberrancies)	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
New iFS*	A	B	B	C	B	C	C	C	A/B	C	C	C	C	C	C	C
Labeled MDS	No	No	No	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes

The four-parameter diagnostic score as described by Della Porta et al.[14] Aberrant myeloid markers, neutrophils and monocytes based on the modified FCSS score. Aberrant myeloid markers as describes in table 2A; more than 2 points per lineage. Aberrant erythroid markers as recommended by the ELNet iMDS-flow, described in Table 2A and the tandem-paper. *Category A 'no MDS-related features', B 'limited number of changes associated with MDS', or C 'features consistent with MDS'. Choice for A or B and B or C depends on the kind and number of aberrancies that are encountered. Note that patients with ≥2 points in the diagnostic score can still be labeled as no MDS by the iFS when there are no other abnormalities.

Table 3 Comparison of dyserythropoiesis as assessed by cytomorphology and flow cytometry.

	By FC (N)	**%**	**By CM (N)***	**%**
Control group	**7/61**	**11**	**6/60**	**10**
Alcohol abuse	0/2	0	0/2	0
Aplastic anemia	0/3	0	0/2**	0
Auto-immune cytopenia	3/10	30	2/10	20
Chronic disease	0/3	0	0/3	0
Eosinophilia	0/1	0	0/1	0
Iron deficiency	4/26	15	0/26	0
Iron incorporation disorder	0/10	0	1/10	10
Medication caused cytopenia	0/3	0	0/3	0
Vitamin B12 deficiency	0/3	0	3/3	100
MDS group	**68/106**	**64**	**81/97*** **	**84**
RCUD	3/4	75	3/3	100
RARS	17/20	85	20/20	100
RCMD	13/23	57	17/20	85
RCMD-RS	19/27	70	26/26	100
RAEB-1	10/14	71	11/12	92
Isolated del(5q)	4/12	33	1/10	10
MDS-U	0/2	0	0/2	0
CMML	2/4	50	4/4	100

Note: *Less than 10% erythroid dysplasia and therefore not enough for diagnosis MDS or >10% and classified MDS according to WHO criteria; **For one aplastic anemia patient there were not enough erythroid cells for proper evaluation; ***Cytomorphological details absent in 9 patients.

Addition of erythroid markers to current MDS-FC scoring systems - Diagnostic score

The original diagnostic score was indicative for MDS in 78/106 MDS patients, and negative for MDS in 53/61 of the control patients (**Figure 1**). Hence, sensitivity and specificity of this diagnostic score were 74% (95% CI: 64%-82%) and 87% (95% CI: 76%-94%), respectively. By erythroid evaluation 64% of MDS patients and 11% of controls revealed erythroid aberrancies by FC (**Table 1**). Erythroid results were added to the diagnostic score as illustrated in **Table 2B.** This led to an upgrade in MDS-FC category in 13 MDS patients and 2 controls. Consequently, the sensitivity and specificity for the diagnostic score including erythroid evaluation were, 86% (95% CI: 78%-92%) and 84% (95% CI: 72%-92%), respectively.

Addition of erythroid markers to current MDS-FC scoring systems - integrated MDS-FC score

With the addition of the erythroid analysis two extra RARS patients, five RCMD patients, four RCMD-RS patients, and one del(5q) patients were now recognized as MDS. The addition of the erythroid analysis did not alter the results for the RAEB-1, MDS-U and CMML patients (**Figure 2 and Table S2**). Results of the original iFS were

C 'compatible with MDS' in 73/106, B 'minor MDS related aberrancies' in 21/106, and A 'not compatible with MDS' in 12/106 MDS patients. Interestingly, each MDS patient not recognized by the original iFS showed only dyserythropoiesis with or without dysmegakaryopoiesis by cytomorphological assessment. In the control group results were A in 40/61 patients, B in 20/61 patients, and C in only 1/61 patients. The calculated sensitivity and specificity of the iFS were 69% (95% CI: 59% to 78%) and 98% (95% CI: 91%-100%), respectively.

In the MDS group 33 patients were not assigned as MDS by the original iFS (**Figure 1**; category A and B). After addition of the erythroid evaluation 12 MDS patients changed from B to C now allocated MDS, and 5 patients in category A were changed to B (limited changes but still no MDS). In total, 21 patients were not allocated as MDS; 7 in category A, 14 in category B.

In the control group one patient was incorrectly identified as MDS (category C). After addition of the erythroid evaluation two extra patients in category B were upgraded to C and thus allocated MDS (**Figure 1)**. Overall, the sensitivity of the iFS increased to 80% (95% CI: 71%-87%) and the specificity showed only a minor decline to 95% (95% CI: 86%-99%).

Figure 1 MDS-FC results in the MDS and control group
The diagnostic score and the integrated MDS-FC score in patients within the MDS group and control group. The arrows demonstrate the patients changing groups after addition of the erythroid evaluation as recommended by the IMDS-flow group. *Flow cytometric results showed minimal dysplastic features, not enough for MDS.

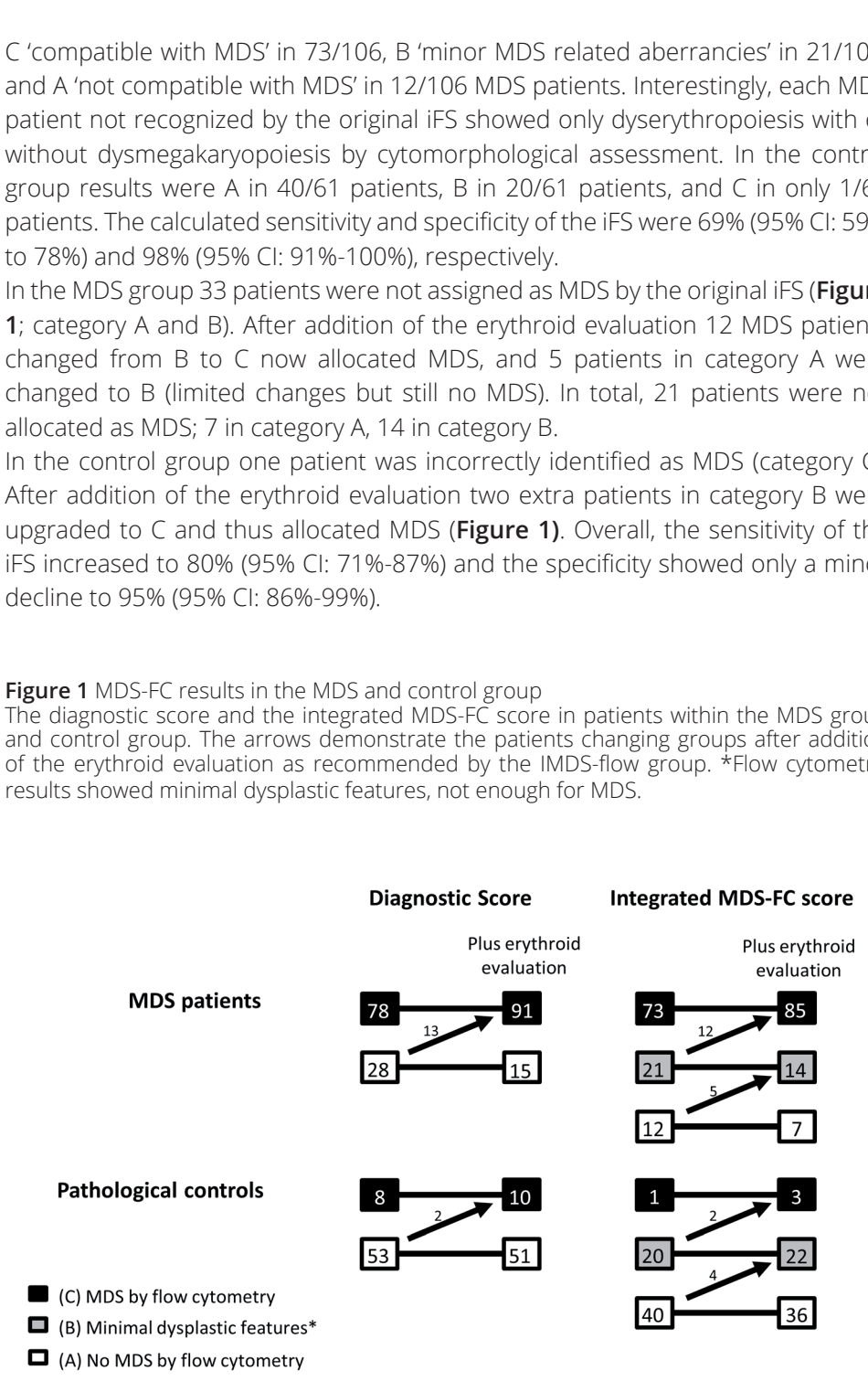
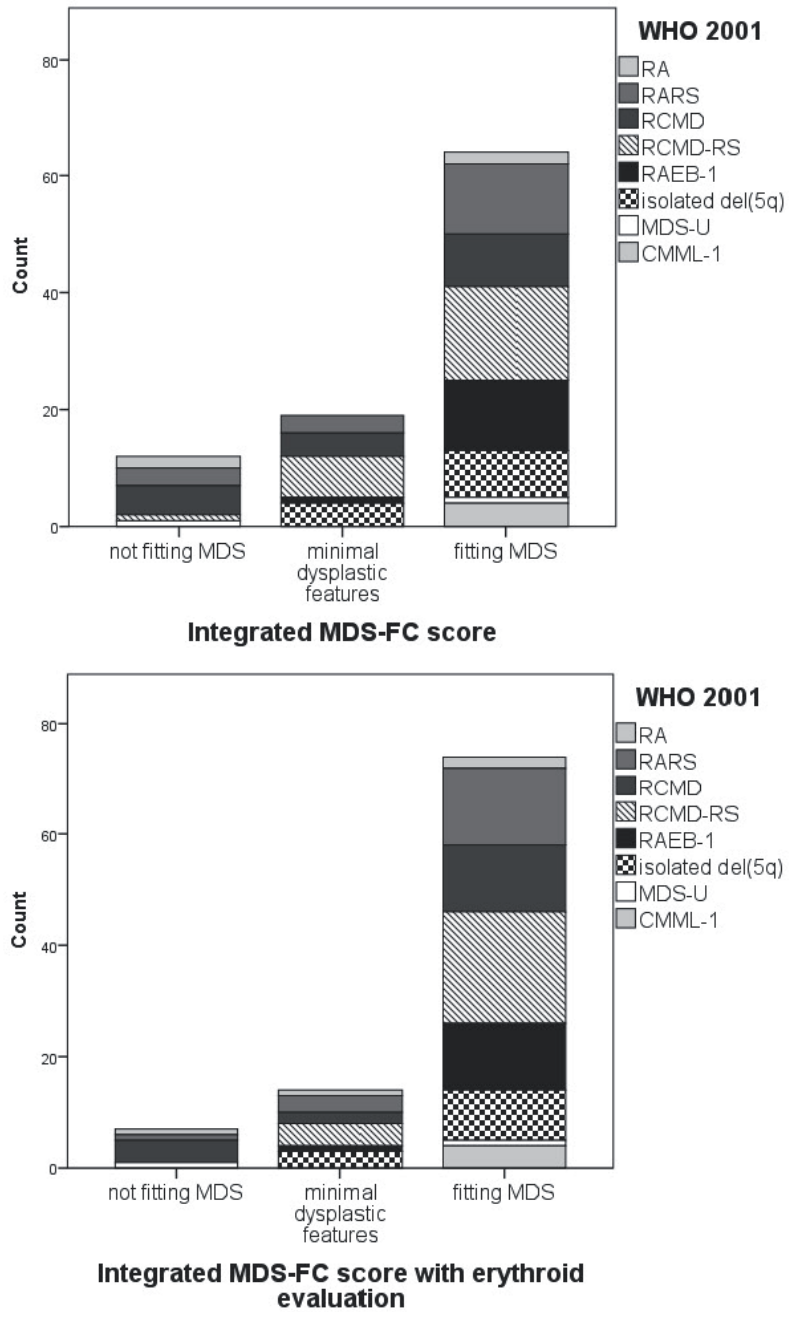


Figure 2 WHO-classifications within different MDS-FC groups
Distribution of WHO-classifications within the original iFS categories, and iFS categories after the addition of the erythroid evaluation. With the addition of the erythroid compartment, patients shift into a higher MDS-FC category. Category A 'no MDS-related features', B 'limited number of changes associated with MDS', or C 'features consistent with MDS'. Absolute patient numbers are provides in the Supplementary files (**Table S2**)



In summary, the sensitivity for both the diagnostic score and the iFS increased significantly after addition of erythroid evaluation. For the diagnostic score, sensitivity increased from 74% to 86%, and the iFS sensitivity increased from 69% to 80%. For both strategies specificity was only marginally affected: 87% to 84% for the diagnostic score and 98% to 95% for the iFS. **Figure 2** illustrates distribution of WHO classifications within the original iFS and after addition of the erythroid evaluation.

Robustness of the MDS-FC results
Interpretation of FC data in MDS is considered to require a high level of expertise. To check solidity of our MDS-FC based conclusions, 25% of the MDS cases were analyzed blindly by an independent MDS-FC expert center (VHJvdV and JtM). The scores were calculated in the same data files. Results for the diagnostic score revealed a concordance of 100% and 89% for the 4-color and 8-color analysis, respectively. Analysis of the iFS revealed a concordance of 89% and 86%, for the 4-color analysis for the 8-color analysis, respectively. Addition of the erythroid evaluation did not influence the concordance of the MDS-FC models.

Discussion

The evaluation of dyserythropoiesis by a flow cytometric (FC) approach is not included in most of today's MDS-FC models. The International/European LeukemiaNet Working Group for Flow Cytometry in MDS (IMDS-flow) proposed a method for evaluation of the erythroid compartment by FC. In the current study we validated the erythroid evaluation and investigated the value of the introduced erythroid evaluation in two previously validated MDS-FC approaches. We analyzed 167 bone marrow aspirates, 106 patients with MDS and 61 cytopenic controls for which the IMDS-Flow erythroid score, diagnostic score, and integrated FC score (iFS) were calculated.[13,16] Originally, the erythroid score was designed as a weighted score. It can also be applied as a numerical score (one point per parameters) in which ≥2 points identifies MDS-associated erythroid aberrancies. Note, the exception made in the tandem-manuscript: if the 2 points are based on the combination of decreased MFI of CD71 and abnormal percentage of CD117, an additional aberrancy is warranted. The latter was not seen in this cohort. Results from the erythroid evaluation confirmed the results of the IMDS-flow report as we showed a strong significant correlation between MDS-associated erythroid aberrancies assessed by FC and MDS. Investigation of the correlations between cytomorphological results and FC results suggested that FC detected less erythroid aberrancies when compared to cytomorphology results. Here,

it needs to be considered that both techniques investigate different aspects. FC mainly evaluates cell surface characteristics, where cytomorphology also evaluates features within the cell such as nuclear bridging. It is unknown whether these dysplastic features result in altered antigen expression. The FC method is however rather specific as for example, it did not report MDS-associated erythroid aberrancies where cytomorphology described dyserythropoiesis in patients with a vitamin B12 deficiency. This indicates that both techniques provide supplementary information, and complement rather than contradict one another.

The goal of the study was to increase the sensitivity of current applied MDS-FC models. Indeed, the addition of the erythroid lineage analysis to the current applied diagnostic score demonstrated an increased sensitivity (from 74% to 86%) without a major loss in specificity (87% to 84%). These results support the findings of Mathis et al, who tested the addition of erythroid evaluation by FC in non-lysed samples (RED score) to the diagnostic score.[22] The combination was analyzed in a cohort of 101 patients (83 MDS patients and only 18 controls) resulting in a sensitivity and specificity of 88% and 89%, respectively. The RED score and the erythroid score described by the IMDS-flow both comprise evaluation of CD36 CV and CD71 CV. Differences were however i) a non-lysed method in the RED score, ii) the addition of the hemoglobin level in the RED score, iii) the added value of percentage of CD117 and iii) added value of expression level of CD71. As illustrated by Mathis and colleagues hemoglobin showed a strong negative correlation with the other markers in the RED-score. Note, hemoglobin might be subject to confounders, e.g. transfusion requirements, and as a non-FC parameter less suitable in a MDS-FC model.

The second diagnostic MDS-FC model evaluated in the current study was the iFS. A more extensive model, comprising the diagnostic score and evaluation of frequently described aberrancies on (im)mature myelo-/monocytic cells. Addition of erythroid markers to this score led to an increased sensitivity (from 69 to 80%), without substantially affecting the specificity (from 98 to 95%). The combination of the iFS with the IMDS-flow erythroid score showed the highest specificity; higher than the other described scores.
Most described MDS-FC scores were designed and validated in large patient cohorts. However, interpretation of results within individual patients can be challenging. To our knowledge the iFS is the only MDS-FC algorithm that has proven its power in individual patients, demonstrated by its high specificity in patients with cytopenias of unknown origin followed over time.[17] After addition of the erythroid lineage evaluation, its specificity remained high and therefore it might be expected that the new model is applicable in individual patient analysis.

To not overcall patients with cytopenia of unknown origin as MDS, one would prefer to apply the most specific model, however in an era where cost-effectiveness is becoming increasingly important a limited panel might be preferred. To improve the four-parameter diagnostic score, Bardet and colleagues advised the addition of CD7 (on myeloid progenitors) and CD56 (on monocytes) to the diagnostic score.[28] Specificity of this adjusted score was 87%, however the sensitivity was low (66%). Here, the addition of selected erythroid markers might improve the sensitivity of FC.

It is suggested to add analysis of mutation in genes involving splicing factors, epigenetic regulators, signal transduction or the cohesion complex, to diagnostic evaluation.[29,30] However, none of the mutations are disease specific, and some mutations appeared to be present in a low frequency in the elderly population.[31] Therefore, more research regarding their role in the diagnostic setting in MDS is warranted. Until then, FC has proven to be a valuable diagnostic tool, able to fill in the gaps where cytomorphology and cytogenetic results are less certain of a diagnosis. It showed to be highly specific in the diagnosis of MDS, thereby able to exclude patients from unnecessary follow-up. MDS-FC is described to be less sensitive in MDS recognition. Our study however, showed that addition of erythroid evaluation to current applied MDS-FC models increased the sensitivity of FC in the detection of MDS. Thereby we postulate that MDS-FC is ready for general clinical application.

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Supplementary files

Table S1A The applied 4-color (panel A) and 8-color (panel B) markers.

	FITC	PE	PerCP	APC
1			**CD45** (2D1) BD	
2	**CD16** (DJ130c) DAKO	**CD13** (L138) BD	**CD45**	**CD11b** (D12) BD
3	**CD34** (8G12) BD	**CD11b** (D12) BD	**CD45**	**HLA-DR** (L243) BD
4	**CD36** (CLB-IVC7) Sanquin	**CD33** (P67.6) BD	**CD45**	**CD14** (MoP9) BD
5	**CD36**	**CD64** (10.1) DAKO	**CD45**	**CD14**
6	**CD15** (MMA) BD	**CD10** (SS2/36) DAKO	**CD45**	**CD34** (8G12) BD
7	**CD34**	**CD117** (104D2) BD	**CD45**	**CD13** (WM15) BD **CD33** (P67.6) BD
8			**CD45**	**CD34**
9	**CD5** (DK23) DAKO	**CD19** (SJ25C1) BD	**CD45**	**CD34**
10	**CD2** (MT910) DAKO	**CD56** (My31) BD	**CD45**	**CD34**
11	**CD13** (WM-47) DAKO	**CD7** (M-T701) BD	**CD45**	**CD34**
12	**CD13**	**CD25** (ACT-1) DAKO	**CD45**	**CD34**
13	**CD71** (Ber-T9) BD	**CD235a** (JC159) DAKO	**CD45**	**CD117** (104D2) Dako

Table S1B

	FITC	PE	PerCP-Cy5.5	PC7	APC	APC-H7	V450	KO
1			**CD34** (8G12) BD	**CD117** (104D2D1) BC			**HLA-DR** (L243) BD	**CD45** (J.33) BC
2	**CD16** (DJ130c) DAKO	**CD13** (L138) BD	**CD34**	**CD117**	**CD11b** (D12) BD	**CD10** (HI10A) BD	**HLA-DR**	**CD45**
3	**CD2** (MT910) DAKO	**CD64** (10.1) DAKO	**CD34**	**CD117**	**IREM2** (UP-H2) IS	**CD14** (MoP9) BD	**HLA-DR**	**CD45**
4	**CD36** (CLB-IVC7) Sanquin	**CD105** (43A3) BL	**CD34**	**CD117**	**CD33** (P67.6) BD	**CD71** (M-A712) BD	**HLA-DR**	**CD45**
5	**CD5** (DK23) DAKO	**CD56** (My31) BD	**CD34**	**CD117**	**CD7** (M-T701) BD	**CD19** (SJ25C1) BD	**HLA-DR**	**CD45**
6	**CD15** (MMA) BD	**CD25** (ACT-1) DAKO	**CD34**	**CD117**	**CD123** (9F5) BD	**CD38** (HB7) BD	**HLA-DR**	**CD45**
7	**CD7** (M-T701) BD	**CD235a** (JC159) DAKO	**CD34**	**CD117**	**CD13** (WM15) BD	**CD71** (M-A712) BD	**HLA-DR**	**CD45**

Per antibody CD number, (clone), and manufacturer are depicted. DAKO: DakoCytomation, Glostrup, Denmark; BD: BD Biosciences, San Jose, CA, USA. BL: BioLegend, San Diego, Ca, USA. Sanquin, Amsterdam, The Netherlands. BC: Beckman Coulter, Miami, FL, USA. IS: Immunostep, Salamanca, Spain.

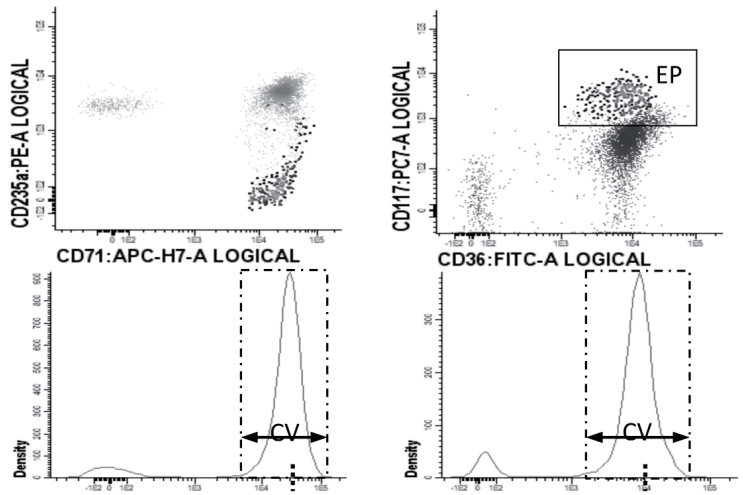
Table 2S Upgraded patients per WHO-Classification after addition of the erythroid evaluation.

WHO-classification	Upgrade A > B 'No MDS'	Upgrade B > C 'MDS'	C 'MDS' after addition erythroid evaluation (group total)
RA	1	0	2 (4)
RARS	2	2	16 (20)
RCMD	1	5	17 (23)
RCMD-RS	1	4	23 (27)
RAEB-1	0	0	13 (14)
Del(5q)	0	1	9 (12)
MDS-U	0	0	1 (2)
CMML	0	0	4 (4)

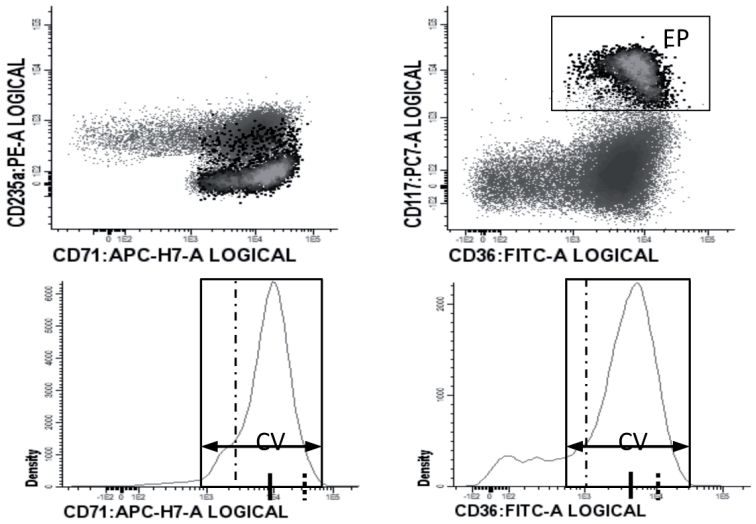
This table provides the absolute patient counts belonging to Figure 2.

Figure S1 Example of a normal bone marrow (A) and a MDS patient (B)
Figure A provides an example of a healthy control. Figure B provides an example of a MDS patient (RCMD-RS) which has an increased percentage of erythroid progenitors (EP). Furthermore, CD71 expression is longer retained during maturation. This is reflected in the increased CV of CD71 and CD36. Finally, the MDS patient shows a decreased MFI of CD71. This patient scores 4 out of 4 points, revealing clear MDS specific dyserythropoiesis as assessed by FC. The dotted lines plotted in figure B are the normal reference subtracted from figure A.

A



B



CHAPTER 6

CONVENTIONAL AND EMERGING DIAGNOSTIC TECHNIQUES DESCRIBE A HETEROGENEOUS LANDSCAPE IN LOW-INTERMEDIATE-1RISK MYELODYSPLASTIC SYNDROMES

A STUDY ON BEHALF OF THE HOVON89 STUDY GROUP

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TO BE SUBMITTED IN PART OF THE CLINICAL HOVON89 STUDY

Abstract

The diagnosis of myelodysplastic syndromes (MDS) is established on conventional cytomorphology, standard karyotyping and recently the presence of a *SF3B1* mutation in relation to ring sideroblasts. Still, MDS remains a very heterogeneous disease with respect to disease characteristics, prognosis and treatment response. To support knowledge of this heterogeneous disease this study combined conventional and emerging diagnostic tools such as high resolution SNP-array, flow cytometry (FC) and targeted next-generation sequencing (NGS) in a well-defined low/intermediate-1 risk MDS cohort of 141 patients. This analysis aimed to identify correlations between the results of these different techniques.

SNP-array detected 55 (41%) abnormal cases, FC detected 105 (81%) aberrant cases, and NGS detected mutations in ≥1 gene in 99 (74%) cases. In 88% of the patients MDS-associated abnormalities were detected when combining the results of FC and NGS. Within the distinct WHO categories, results were very heterogeneous with respect to FC aberrancies and mutations identified by NGS. SF3B1, TET2, DNMT3A and ASXL1 were the most commonly affected genes. There was a minor but significant positive correlation between the number of FC aberrancies and the number of mutations detected by NGS. Mutations in transcription factors and epigenetic regulators correlated with an aberrant progenitors, granulocytes and monocytes. This study demonstrates that MDS-FC and NGS identify a divers landscape within the well-established WHO categories. Addition of these techniques in the standard work-up in MDS might improve diagnostic and prognostic models, and might assist prediction of treatment response in the future.

Introduction

Myelodysplastic syndromes are a heterogeneous group of clonal hematopoietic bone marrow disorders, characterized by cytopenias, dysplasia and increased risk of leukemic transformation. The diagnosis is established based on conventional diagnostic criteria such as the presence of (>10%) dysplasia in one or more cell lineages, the presence of >15% ring sideroblasts (or >5% in case of a SF3B1 mutation) as assessed by cytology, or the presence of MDS-associated cytogenetic abnormalities.[1–3] Based on these parameters the WHO classification assigns patients to separate diagnostic categories. Although this strategy is straightforward, patients within the same category can still be very heterogeneous with respect to symptoms, prognosis and response to therapy. In an attempt to identify more homogeneous patients categories, diagnostic guidelines for MDS established by the European LeukemiaNet (ELN) recommend the addition of flow cytometry (FC), and suggest additional molecular techniques to support the diagnosis of MDS.[4 5]

With the use of FC, myeloid and B cell progenitors, erythroid and maturing myeloid cell subsets can be evaluated. This allows detection of multilineage aberrancies within patients with unilinear dysplasia by cytomorphology, and detection of aberrant myeloid progenitor cells in patients with low blast percentages as assessed by cytomorphology.[6–8] It was shown, that multiple aberrancies correspond with higher risk IPSS-R categories and with a worse outcome within the lower IPSS-R groups.[9,10] Additionally, presence of aberrant myeloid progenitor cells corresponds with poor treatment response in low and high risk MDS, with growth factors and azacitidine, respectively.[11,12]

The role of next generation sequencing (NGS) in the diagnostic strategy in MDS is very promising. Large scale genomic screening studies found mutations that describe a divers mutational landscape in MDS.[13] Mutations are found in genes coding for splicing factors, epigenetic regulators, transcription factors, cohesin complex components, and signal transduction molecules.[14,15] Studies regarding correlations between specific mutations and phenotypical characteristics in myeloid disease, i.e. MDS/AML, are currently ongoing. For example, bi-allelic mutated CEBPA correlates with expression of CD7 on myeloid progenitors in AML.[16,17]

The combined use of FC and targeted NGS might help to improve the diagnosis of cytopenic patients suspect for MDS, may add prognostic information, and ultimately assist in choosing the most appropriate therapy. Therefore, this study displayed the results of conventional and emerging diagnostic techniques within

the well-established WHO classifications, in a well-defined low-intermediate-1 risk MDS population within a prospective clinical trial. Moreover, this analysis aimed to identify correlations between the results of these techniques, to support knowledge aid future research on pathophysiological mechanisms to identify homogenous patient categories in this heterogeneous disease. More homogenous patients in respect to prognoses and treatment response that aid clinical decision making.

The study was conducted within the HOVON89 trial: a prospective randomized phase II study, that investigates the efficacy of lenalidomide without (study arm A) or with (study arm B) the addition of growth factors. This trial is registered at www.trialregister.nl as NTR1825; EudraCT nr.: 2008-002195-10. The samples in the current study were taken before treatment with lenalidomide. The results of this study will be discussed separately. Therefore, no correlations with treatment response to design a predictive model for response is discussed within the current report.

Methods

Study design HOVON 89

The HOVON 89 study is a prospective open label multicenter phase II study that evaluates the effect of lenalidomide (Revlimid) with or without growth factors. Adult low/intermediate-1 risk MDS patients (according to the IPSS[18]) were randomized between lenalidomide alone (arm A) or lenalidomide with a standardized regimen of growth factors (arm B). In addition, add-on studies comprised analysis by SNP-array, FC and NGS. The research was conducted according to the Declaration of Helsinki; the research program was approved by the local ethics committees and written informed consent was obtained (www.trialregister.nl; NTR1825; EudraCT nr.: 2008-002195-10).

This report describes the analysis of the samples that were taken prior to treatment. Patients were accrued between May 2009 and September 2015. Cytomorphology results were centrally reviewed and assessed according to the WHO 2001 (at study design the WHO 2008 and 2016 classifications were not yet established).[1] Karyotyping and interphase FISH were performed in local certified cytogenetic laboratories. After central review, the data were collected at the HOVON data center, Rotterdam, The Netherlands. FC analysis was performed in the VU University Medical Center, Amsterdam, The Netherlands. SNP-arrays and NGS were performed at the Radboud University Medical Centre, Nijmegen, The Netherlands.

Patients

Two-hundred patients were randomized between study arm A and B. After randomization 16 patients were excluded by the review committee as they did not meet the inclusion criteria. One-hundred and eighty-two patients gave informed consent for the additional studies as described within this study. For these patients bone marrow samples to perform SNP-array, FC, and NGS were requested at the local sites. In 141 cases material was received (**Table 1**).

Karyotyping, FISH and SNP-array

Routine karyotyping was performed using standard methods and at least 20 metaphases were analyzed according to the guidelines of the European cytogenetic association for acquired cytogenetics. Karyotypes were described according to the standardized ISCN2013 nomenclature system.[19] In patients with less than 20 normal metaphases or less than 10 metaphases in the presence of a clonal abnormality, additional interphase FISH analyses for the detection of -5, 5q-, -7, 7q- and +8 were performed using commercially available probes (Abbott Laboratories, Abbott Park, Illinois, USA).

Genomic array profiling was carried out in a central laboratory using the CytoScan HD array platform (Affymetrix, Inc., Santa Clara, CA, USA). Hybridizations were performed according to the manufacturer's protocols. The data were analyzed using the Chromosome Analysis Suite software package (Affymetrix), using annotations of genome version GRCh37/hg19.

For a comprehensive interpretation of the SNP-based genomic array profiling data we used criteria previously described.[20] According to these guidelines all segments larger than 5 Mb (resolution of conventional karyotyping), regardless of gene content, were denoted as true aberrations. Segments smaller than 5 Mb, were included only if they coincided with known cancer genes (http://cancer.sanger.ac.uk/cancergenome/projects/census/date of accession November 2012). Since paired control DNA was not used, alterations that coincided with normal genomic variants were excluded. For this approach the publicly available database 'Database of Genomic Variants' (http://projects.tcag.ca/variation) and an in-house database in which CNVs are stored from ~1,000 healthy individuals run on the same CytoScan HD platform were used. Regions of CNLOH were only considered if they were >10 Mb in size and if they extended towards the telomeres of the involved chromosomes, as reported in.[21] Focal CNAs in the immunoglobulin genes were excluded. All the data were also visually inspected to define alterations present in a lower proportion of cells (mosaics), and to eliminate alterations reported in regions with low probe density. Only aberrations fulfilling the above criteria were

Table 1 Patient characteristics

	Patient count
Study inclusion	200
Approval additional studies	182
Material received	141
FC performed	129
NGS performed	133
Age in years (median and range)	71 (38-75)
Gender (m/f)	80/61
Mutations 0 1 2 3 4 5	133 34 35 36 21 5 2
Mutational categories involved 0 1 2 3 4	133 34 42 42 14 1

included in the genomic profiles, and were described according the standardized ISCN 2013 nomenclature system.

Flow Cytometry

Flow cytometry was conducted according to the guidelines concerning recommended methods for cell sampling, handling and processing, published by the ELNet Working Party on Flow Cytometry in MDS.[22] Samples were processed within 24 hours. Mature erythrocytes were lysed using ammonium chloride-based erythrocyte lysing solution. Nucleated cells were pre-incubated with human serum immunoglobulins before staining. The antibody panels are provided in the **supplementary file**.

Due to technical developments a 4-color method was applied between 2009 and 2012 (FACSCalibur; BD Biosciences, San Jose, CA), and an 8-color method (FACSCanto II; BD Biosciences) between 2012 and 2014. A total of 100,000 leukocyte events were acquired, including a minimum of 250 CD45diminished/CD34+ events.[23] Data from the 4-color method were analyzed with CellQuestPro (BD Biosciences); data from the 8-color method were analyzed with Infinicyt

software (Cytognos, Salamanca, Spain). Results were discussed within an expert MDS-flow cytometry team, with ≥3 researchers. Reference values for marker expression were established within normal age-matched controls; values two-times the standard deviation above or below normal were considered aberrant.[7]

Integrated MDS-flow cytometric score (iFS)

The integrated flow cytometric score (iFS) was applied to summarize MDS-FC results [22]. This score integrates the four-parameter diagnostic score described by Della Porta and Ogata and separate cell lineage evaluations largely based on Wels et al.[7,23] The diagnostic score comprises the percentage of CD34 positive cells, CD45 expression of myeloid progenitors, SSC of the granulocytes, and percentage of progenitor B cells. The erythroid lineage evaluation included evaluation of: CD71 expression and the coefficient of variation (CV), CD36 CV and percentage of CD117 within the CD45diminished-negative erythroid cell compartment.[24,25]

If there were no flow cytometric abnormalities the patient was categorized as 'no MDS-related features'. If one cell compartment (progenitor, erythroid, or myeloid) was affected, the patient was categorized as 'limited number of changes associated with MDS', and if ≥2 cell lineages were affected the patient was categorized as 'features consistent with MDS'. Additionally, patients with ≥2 abnormalities according to the four-parameter diagnostic score altered the allocation to above mentioned categories: a patient within the 'no MDS-related features' category became 'limited number of changes associated with MDS'; and patients categorized as 'limited number of changes associated with MDS' with ≥2 abnormalities according to the diagnostic score were now categorized as 'features consistent with MDS'.[25]

Next generation sequencing

DNA was isolated from bone marrow mononuclear cells using nucleospin columns (BioKe, The Netherlands). Sequencing was performed using Roche 454 technology, largely as described.[26] Amplicons covering all relevant regions in 20 genes frequently affected in MDS (ASXL1, CBL, CSF1R, DNMT3A, ETV6, EZH2, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, SF3B1, SRSF2, TET2, TP53, U2AF1 and ZRSR2) were designed (Supplemental table S2. Primers contained a 10 bp barcode, Supplemental files). Pools were prepared for the emulsion PCR (emPCR) step. First, the pools were diluted to a concentration of 4x106 molecules per µl. Secondly, the libraries were processed using the GS FLX Titanium Series Lib-A SV method (Roche Applied Science). Forward (A beads) and reverse (B beads) reactions were carried out using 2,000,000 beads per emulsion oil tube. The copy per bead ratio used was 2.1. The amplification reaction, breaking of the emulsions and enrichment of beads carrying amplified DNA was performed using the workflow as

recommended by the manufacturer. However, A beads and B beads were separately processed during breaking and enrichment processes. Finally, samples were loaded on an 8-lane PicoTiterPlate (PTP) on the Genome Sequencer FLX System instrument (Roche Applied Science). All data were generated using the GS FLX Sequencer Instrument software version 2.3. Image processing and amplicon pipeline analysis was performed using default settings of the GS RunBrowser software version 2.3 (Roche Applied Science).

Amplicons covering relevant regions of CSF1R, SF3B1, SRSF2 and U2AF1 (See Supplemental table S3 for primers) were also sequenced using Ion Torrent semiconductor technology, as described.[27] PCRs were performed using conventional sequencing primers in a fully automated robotic work flow. PCR amplicons were subsequently pooled and sheared to 200-300 bp. Library preparation was performed in an automated fashion on a MicroLab Starlet Replicator Robot (Hamilton) by using the Ion Plus fragment library kit in combination with the Ion XpressTM barcode adapters 1–96 kit (both Life Technologies). Emulsion PCRs were performed on an Ion OneTouch system (Ion OT2 instrument, Life Technologies) using the Ion PGM Template OT2 200 kit. Enrichment of template-positive Ion sphere particles (ISPs) was performed on a OneTouch ES system (Life Technologies). The percentage of template-positive ISPs was measured with the use of the Ion Sphere Quality Control kit (Life Technologies) and a Qubit 2.0 Fluorometer (Invitrogen). Subsequently, ISPs coated with template were loaded on Ion 318TM sequencing chips (Life Technologies). The chips were sequenced on the PGM, with the use of the Ion PGM sequencing 200 kit version 2.

Sequence alignment and variant detection was performed using the SeqNext module of Sequence Pilot software, version 4.1.2 (JSI medical systems, Germany). Within SeqNext, the sequencing reads were mapped to defined ROIs, and variant calling was performed using user-defined settings. Analysis parameters in combination with selective procedures were used to ensure high coverage and high sensitivity, thus taking specific sequencing technology–based limitations into account (e.g., bases with low base call quality and homopolymer topics). For the detection of variants, filters were set to display sequence variants occurring in more than 10% of bidirectional reads per amplicon. Mutations within introns and single nucleotide polymorphisms (SNPs; dbSNP build 131) were discarded.

Statistics

Results from FC and NGS were visualized in bar plots, and compared using the appropriate statistical tests. Correlations of scale outcomes between two or more patient groups were investigated by the Mann-Whitney U test. Correlations between two ordinal (three or more categories) outcomes were analyzed applying a chi-square test. P-values below 0.05 were considered statistically significant. Correlation between two scale-outcomes were analysed by the Spearman Correlation test, were a correlation coefficient above 0.7 was considered a strong correlation. The analyses were performed using PASW Statistics version 20.0 (SPSS, Chicago, IL).

Results

Out of the 182 patients who gave approval for further analysis for additional laboratory studies, material from 141 patients was actually received. In 129 (91%) cases FC was performed (other cases concerned inadequate samples) or technical issues i.e. not enough events were acquired); 133 (94%) samples were suitable for SNP-array and NGS (not enough material in the remaining set). Median age of the 141 evaluated patients was 71 years (range 38-85 years), 80 were male (57%; **Table 1**).

Diagnosis of MDS according to WHO 2001

Accrual of patients in the clinical trial started before the WHO criteria of 2008 and 2016 were established. Therefore, patients were classified according to the WHO 2001 criteria.[1] Diagnoses were: 3 patients with MDS-RA, 21 with MDS-RARS, 31 with MDS-RCMD, 34 with MDS-RCMD-RS, 25 with MDS and isolated deletion of chromosome 5q, 3 with MDS-unclassifiable, 17 with MDS-RAEB-1 and 7 patients with a CMML-1 **(Figure 1A)**.

SNP-array had a higher diagnostic yield than conventional karyotyping

Besides standard karyotyping and FISH analysis, SNP-based genomic arrays were performed. SNP-array was performed in 133 (94%) patients. This is an extended analysis of the study as previously published by Stevens-Kroef et al.[20] In patients for whom both techniques were performed, a higher diagnostic yield for SNP-array was observed resulting in 55 (42%) abnormal cases, while an abnormal karyotype was found in only 35 (27%) patients. In 5 cases when karyotyping failed or in case of an inadequate number of metaphases, SNP-based array analysis was successful. Of interest, in 26 (20%) patients (including 18 with normal karyotype) small focal copy number abnormalities or regions of copy neutral loss of heterozygosity abnormalities were found that were out of the scope of karyotyping and FISH. The SNP-array showed a wide variety in type of mutations within a WHO category (**Figure 1B**). A deletion in chromosome 5q was the most observed mutation (20%), followed by a deletion in chromosome 4q (6%) and 7q (4%). A deletion of the Y-chromosome, which is frequently described as MDS-associated but also as age-associated, is rare in this cohort (1%). One patient had a monosomy 7 as single mutation. There were no complex karyotypes defined as 3 or more mutations.

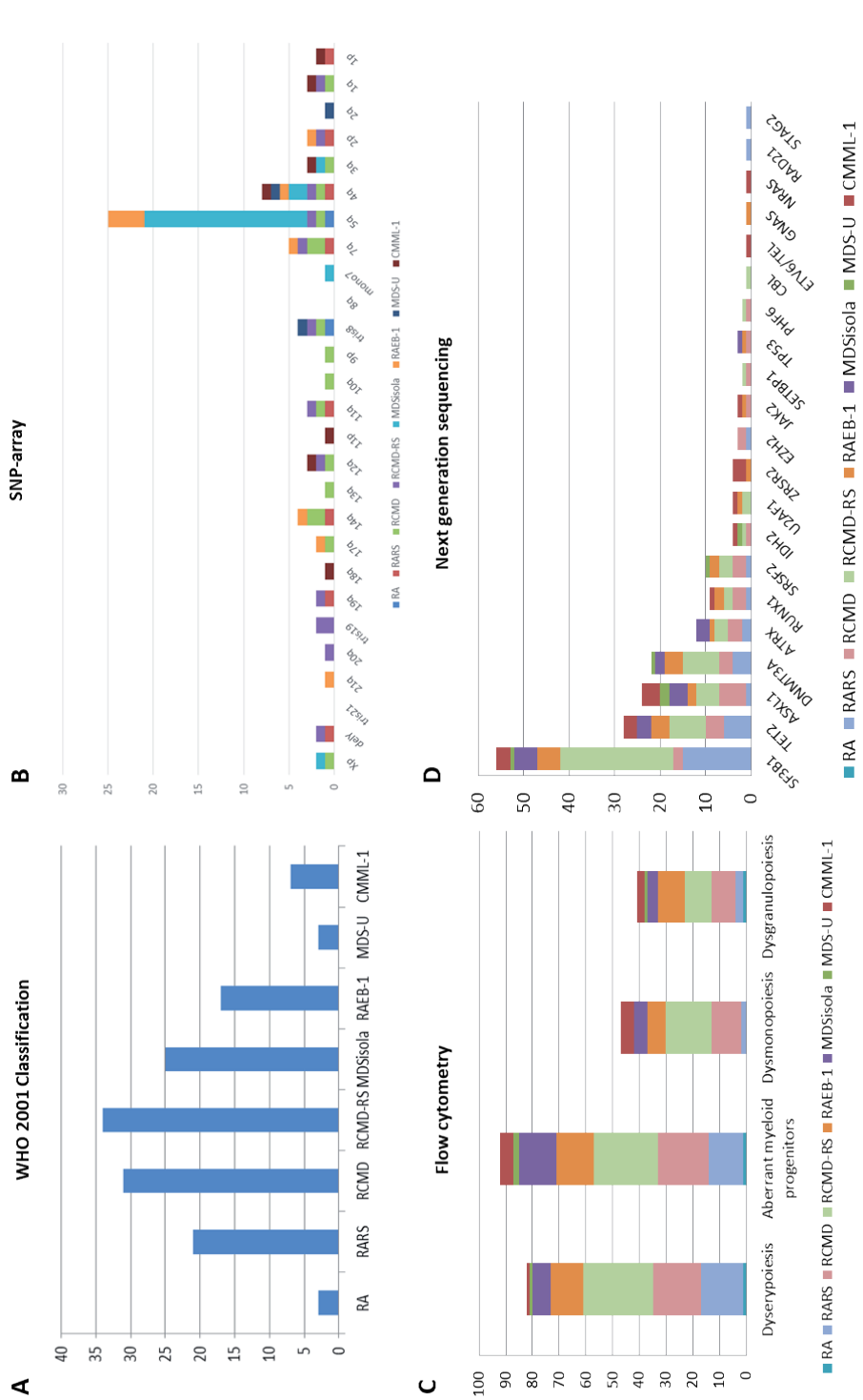

Figure 1 The result of 141 patients per applied tool: cytomorphology (A), SNP-array (B), flow cytometry (C) and next generation sequencing (D). The frequency per diagnostic tool is represented on the y-axes.

Flow cytometry detects multilinear aberrancies in unilinear dysplasia cases

Flow cytometry was adequately performed in 129 (91%) patients. Analyses of the erythroid, progenitor and myeloid cell compartment showed MDS-associated aberrancies in 105 (81.4%) patients **(Figure 1C)**. Only minimal aberrancies were found in 17 (13.2%) patients, and 7 (5.4%) patients were normal according to integrated FC score analyses.

Most MDS patients (78%) showed aberrant myeloid progenitors, a feature that cannot be assessed by cytomorphology. Erythroid dysplasia-associated aberrancies (most frequently found in MDS by cytomorphology) were also frequently found (86%). The presence of ring sideroblasts (irrespective to specific WHO category) correlated with an aberrant erythroid cell compartment (p = 0.015). There was no correlation between lineage aberrancies as assessed by FC and the WHO 2001 category.[25] To illustrate this, in 23/24 RA(RS) cases with unilinear dysplasia based on the WHO2001 criteria for cytomorphology, FC showed multilinear aberrancies. In one additional case cytomorphology described only dyserytropoiesis, whereas FC showed no erythroid aberrancies but dysgranulopoiesis.

Patients classified as MDS-unclassifiable show <10% dysplasia by cytomorphology per definition. Here, two out of three MDS-U patients showed multilineage FC-aberrancies; one other showed only aberrant monocytes by FC. This means, that here FC can help to underline the diagnosis of MDS.

Next Generation Sequencing confirmed results of previous MDS studies

Sequencing was performed in 133 (94%) patients. Within this MDS patient cohort SF3B1 (43%) and TET2 (21%) were the most commonly affected genes, followed by DNMT3A (17%) and ASXL1 (16%; **Figure 1D**). As expected, mutations such as TP53, NRAS, KRAS and FLT3, were rare in this IPSS low-intermediate-1 cohort. Mutations concerned a splicing factor in 74 (56%) patients, an epigenetic regulator in 64 (48%) patients, a transcription factor in 27 (20%) patients, and in 5 (4%) patients there was a mutation in a signal transduction gene. A single mutation was present in 35 patients (26%), more than one mutation was present in 64 (48%) patients, of whom 57 patients had a mutation in more than one mutational category (**Table 1**).

Our data confirmed the correlation between the presence of ring sideroblasts and mutations in splicing factors (p<0.001). As reported before this correlation was not 100%: some patients with ring sideroblasts lacked a SF3B1 mutation and some patients with a SF3B1 mutation lacked ring sideroblasts. Note, in our cohort four patients with ring sideroblasts, without an SF3B1 mutation had another splicing factor mutation, i.e. a SRSF2 mutation.

Except for one patient, a mutation in a splicing factor was restricted to one type of splicing factor mutation confirming that mutations within this class are mutually exclusive (Figure S1). This was not the case for mutations in epigenetic regulators (i.e. DNMT3A), as they often coincided with other epigenetic regulator mutations. Furthermore, RUNX1 and JAK2 mutations were never seen as single mutations.
As described in the WHO 2016 criteria for MDS: 80% of CMML patients have a mutation in SRSF2, TET2 or ASXL1. In this cohort, all tested CMML patients had a mutation detected by NGS: 5/7 in ASXL1, and 3/7 in TET2 (**Figure S1**).

Flow cytometry and next generation sequencing identify MDS-associated abnormalities
In 122 patients both FC and NGS were performed; in 7 additional patients there was only FC data and in 11 additional patients there was only NGS data. In one patient neither FC or NGS could be performed. Flow cytometry identified 105/129 (81%) patients with MDS-associated aberrancies. Next generation sequencing revealed at least one mutation in 99/133 (74%) patients (**Table 1**). In the 122 patients where both techniques were performed, 80 (66%) patients were classified as MDS according to FC with additional MDS-related gene mutations. In 18 (14%) patients with MDS according to FC, no mutations by NGS were observed. In 12 (10%) patients with MDS mutation were present by NGS but no aberrancies were observed according to FC. Finally, 12 (10%) MDS patients were not recognized by either FC or NGS.

By combining the results of both techniques 124/140 (89%) patients showed MDS-associated abnormalities. The remaining 16 patients included 2 RA patients, 1 RARS patient, 6 RCMD patients, 1 RCMD-RS patient, 5 patients with an isolated deletion of chromosome 5q, and 1 RAEB-1 patient. In the latter FC was not performed; this patient showed an abnormal karyotype and was therefore recognized as MDS. Besides this RAEB-1 patient and the patients with an isolated deletion of chromosome 5, the patients that were not recognized by FC and/or NGS showed also no abnormalities according to SNP-array. Thus, in 10 patients the diagnosis MDS was established solely based on the presence of a cytopenia and dysplasia by current standard conventional cytomorphology criteria.

Mutations in transcription factors correlate with an aberrant phenotype
A heterogeneous landscape of FC aberrancies and anomalies as assessed by SNP-array and NGS was identified in this low/intermediate-1 risk MDS population (summarized in **Figure S1**). In 122 patients we were able to investigate correlations between FC aberrancies and NGS. There was a minor but significant positive correlation between the number of FC aberrancies and the amount of mutations detected by NGS (r = 0.24; p=0.01). The number of cases per anomaly found by SNP-array were too small to perform proper statistics.

Due to small patient numbers per mutational subcategory, correlations between aberrant cell compartments by FC and mutations were analyzed per mutational category. There was a significant correlation between mutations in transcription factors and aberrant myeloid progenitors, granulocytes and monocytes according to FC (**Table 2**). There was also a significant correlation between mutations in an epigenetic regulator and presence of aberrant myeloid progenitors, and a correlation between signal transduction factors and aberrant granulocytes. FC did not correlate with splicing factor mutations.

Table 2 Correlations between FC and NGS

	Erythroid	Myeloid progenitor	Granulocytic	Monocytic
Splicing	ns*	ns	Ns	ns
Epigenetic	ns	R 7.95; P = 0.005	Ns	ns
Transcription factors	ns	R 6.89; P = 0.009	R 3.96; P = 0.05	R 5.08; P = 0.02
Signal transduction	ns**	ns**	R 5.32; P = 0.02**	ns**

Abbreviations NS: not significant; *Chi-square test: correlation coefficient; p=value; df(4).
** Note, based on N= 3 cases

No correlation between flow cytometric aberrancies and the IPSS score
Since this study initiated patient accrual in January 2008, we applied the IPSS instead of the now widely applied IPSS-revised prognostic scoring system. Patients with low/intermediate-1 risk MDS were included. Results of FC and NGS were rather heterogeneous within the risk groups. No correlations were observed between the iFS and IPSS or a particular mutational group and IPSS. This might suggest that both techniques may have prognostic value within IPSS subgroups.

Discussion

Myelodysplastic syndromes (MDS) are a heterogeneous group of bone marrow diseases. Heterogeneity is reflected by the WHO classification of MDS as well as by the landscape of flow cytometric (FC) and molecular aberrancies. In addition, the diversity in the natural course of the disease and response to current treatment modalities emphasize huge variability in the spectrum of bone marrow failure

syndromes such as MDS. As more treatment modalities become available the need for better and more directive diagnostic and prognostic tools is growing which might select patients for precision medicine. The combined use of techniques such as SNP-array, FC and targeted next-generation sequencing (NGS) might help to i) improve the diagnosis of patients with MDS; ii) add prognostic information, and iii) ultimately assist in choosing the most appropriate therapy. The current study addressed the role of these emerging technologies in diagnostics by the description of a cohort with 141 low and intermediate-1 risk MDS patients, treated within the HOVON89 study protocol. Patients were analyzed by morphology, conventional karyotyping, SNP-array, FC and NGS.

An abnormal karyotype was found in 35 patients. SNP-array detected 55 (41%) abnormal cases. FC detected 105 (81%) aberrant cases. NGS detected mutations in 99 (74%) patients. By adding the results of FC and NGS 88% patients showed MDS-associated abnormalities. Interestingly, in a total of 11 patients, the diagnosis MDS was established solely based on presence of a cytopenia and >10% dysplasia by standard conventional cytomorphology, without other criteria. This is an interesting group for long-term follow-up. One might expect that these patients have a higher overall survival than patients with multiple aberrancies or mutations.

The correlation between standard karyotyping and SNP-array was previously published.[20] Here we extended this analysis confirming that there is a higher diagnostic yield for SNP-array compared to standard karyotyping, except for mutations without loss of heterogeneity. Seventy-five percent of these only low to intermediate-1 risk MDS patients had one or more mutations (**Figure S1**), somewhat lower than reported in previous research reports where 80-90% was reported in low to high risk MDS patients. Sequencing was performed in 133 (94%) patients. In 99 (74%) patients a mutation was present. Within this low-intermediate-1 risk MDS patient cohort SF3B1, TET2, DNMT3A and ASXL1 were the most commonly affected genes. We found a mutation in a splicing factor in 56% of the cases, corresponding with the previously described 60% in a similar cohort of lower risk MDS.[2] In line with previous reports, the majority of patients with splicing factor mutations showed restriction to one type of splicing factor mutation. This was not the case for mutations in epigenetic regulators, were epigenetic regulators appear to coincide. In addition, DNMT3A, a mutation commonly seen as a single mutation in clonal hematopoiesis of indeterminate potential (CHIP), often observed in the elderly, was rarely seen as single mutation in this cohort (only in one case). This mutation may be of limited leukemic potential as a single mutation co-occurs with other mutations in MDS. RUNX1 (N=12) and JAK2 (N=3) were never seen as single mutation as previously described.[13,28]

Sato and colleagues described 'pre-leukemic stem cells' that will either propagate to a malignant state or will never lead to leukemic evolution.[29] Of note, the patients in this cohort are all refractory or are unlikely to respond to erythropoietin at inclusion in the study. This means that our analysis was not performed in *de novo* MDS, which may imply the presence of additional mutation due to clonal evolution. This may explain why high-risk mutations (mutation correlated with poor survival) were relatively frequent in our cohort, i.e. ASXL1 was found in 21 (16%) cases as compared to other studies. Also other mutations such as TP53, EZH2, ETV6, RUNX1 and SRSF2 were more frequently seen then previously reported in a low/intermediate-1 risk cohort.[2] Identification of type, function, and moment of occurrence of a certain mutation with or without abnormal phenotype will give more answers to why MDS is such a heterogeneous disease.

As described in the WHO 2016 criteria: 80% of CMML patients have a mutation in SRSF2, TET2 or ASXL1. In this cohort with only a small subset of CMML patients, all patients had a mutation in one of these genes (**Figure S1**). All CMML patients had multilineage aberrancies by flow cytometry.

Cytopenias do not always correlate with the lineage affected with dysplasia. In addition, our study showed that the lineage affected by dysplasia as assessed by cytomorphology did not show an 1:1 correlation with FC results (figure 1C). Most patients with unilineage dysplasia according to cytomorphology could have multilineage aberrancies by FC. Phenotype is a result of genotype. Therefore, we expected to find correlations between FC aberrancies and mutations. In 122 patients, we were able to investigate correlations between FC aberrancies and NGS, irrespective of WHO classifications. First, we found a minor but significant positive correlation between the number of FC aberrancies and the number of mutations detected by NGS. This confirms the phenotype-genotype link which might imply a disease-risk association. As most of these were all low risk patients, those patients with multiple aberrancies and additional mutations might identify a higher risk disease despite the low risk IPSS category, or clonal evolution during the course of the disease since most of these patients were not analyzed upfront but after being refractory to erythropoietin as first line of treatment. [31] Note, that in some subcategories patient numbers were small (i.e. only 3 patients with a mutation in a signal transduction gene). Therefore, patients were analyzed in (sub)categories to increase group size. Overall, mutations in transcription factors correlated with an aberrant immunophenotype (aberrant myeloid progenitors, granulocytes and monocytes). Furthermore, mutations in epigenetic regulators correlated with aberrant myeloid progenitors. Because of the heterogeneity observed, large patient numbers are required per mutational category to draw

solid conclusions. Large, multicenter patient cohorts are required to validate the correlations that were found, and to identify possible new correlations. Overall, these results might reflect the heterogeneity in disease manifestation, prognosis and response to therapy in MDS patients.

We demonstrated that MDS-FC and NGS were complementary to cytomorphology (the gold standard) in a diagnostic setting. Patients classified as MDS-U, a challenging diagnosis by cytomorphology (lacking dysplasia), showed clear MDS-FC aberrancies (even multilinear dysplasia in one case).

In summary, this study reveals very heterogeneous with respect to FC aberrancies and SNP-array and NGS abnormalities within the distinct WHO categories. By combining the results of FC and NGS, 88% of patients showed MDS-associated abnormalities. There was a significant positive correlation between the number of FC aberrancies and the number of mutations detected by NGS. Mutations in transcription factors and epigenetic regulators correlated with presence of aberrant myeloid progenitors, granulocytes and/or monocytes by FC. With the availability of new, more advanced diagnostic tools, the classification of MDS is still heavily weighted on cytomorphology and cytogenetics. Although in the WHO 2016 criteria assessment of *SF3B1* in patients with ring sideroblasts is now added, this is just the beginning of improvement of MDS classification. MDS-FC can be used to identify multilineage aberrancies in single lineage dysplasia by cytomorphology. The diagnostic value of NGS is still under consideration. Mutation are described as frequent, yet their impact on therapy and prognosis is unknown.[32] This study demonstrates that MDS-FC and NGS are able to identify a divers landscape within the well-established WHO categories. More research on the impact of these techniques and the prediction of overall survival and treatment response is ongoing.

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Conflict of interest statement: none declared.

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Supplementary files

Table S1A The applied 4-color (panel A) and 8-color (panel B) markers.

	FITC	PE	PerCP	APC
1			**CD45** (2D1) BD	
2	**CD16** (DJ130c) DAKO	**CD13** (L138) BD	**CD45**	**CD11b** (D12) BD
3	**CD34** (8G12) BD	**CD11b** (D12) BD	**CD45**	**HLA-DR** (L243) BD
4	**CD36** (CLB-IVC7) Sanquin	**CD33** (P67.6) BD	**CD45**	**CD14** (MoP9) BD
5	**CD36**	**CD64** (10.1) DAKO	**CD45**	**CD14**
6	**CD15** (MMA) BD	**CD10** (SS2/36) DAKO	**CD45**	**CD34** (8G12) BD
7	**CD34**	**CD117** (104D2) BD	**CD45**	**CD13** (WM15) BD **CD33** (P67.6) BD
8			**CD45**	**CD34**
9	**CD5** (DK23) DAKO	**CD19** (SJ25C1) BD	**CD45**	**CD34**
10	**CD2** (MT910) DAKO	**CD56** (My31) BD	**CD45**	**CD34**
11	**CD13** (WM-47) DAKO	**CD7** (M-T701) BD	**CD45**	**CD34**
12	**CD13**	**CD25** (ACT-1) DAKO	**CD45**	**CD34**
13	**CD71** (Ber-T9) BD	**CD235a** (JC159) DAKO	**CD45**	**CD117** (104D2) Dako

Table S1B

	FITC	PE	PerCP-Cy5.5	PC7	APC	APC-H7	V450	KO
1			**CD34** (8G12) BD	**CD117** (104D2D1) BC			**HLA-DR** (L243) BD	**CD45** (J.33) BC
2	**CD16** (DJ130c) DAKO	**CD13** (L138) BD	**CD34**	**CD117**	**CD11b** (D12) BD	**CD10** (HI10A) BD	**HLA-DR**	**CD45**
3	**CD2** (MT910) DAKO	**CD64** (10.1) DAKO	**CD34**	**CD117**	**IREM2** (UP-H2) IS	**CD14** (MoP9) BD	**HLA-DR**	**CD45**
4	**CD36** (CLB-IVC7) Sanquin	**CD105** (43A3) BL	**CD34**	**CD117**	**CD33** (P67.6) BD	**CD71** (M-A712) BD	**HLA-DR**	**CD45**
5	**CD5** (DK23) DAKO	**CD56** (My31) BD	**CD34**	**CD117**	**CD7** (M-T701) BD	**CD19** (SJ25C1) BD	**HLA-DR**	**CD45**
6	**CD15** (MMA) BD	**CD25** (ACT-1) DAKO	**CD34**	**CD117**	**CD123** (9F5) BD	**CD38** (HB7) BD	**HLA-DR**	**CD45**
7	**CD7** (M-T701) BD	**CD235a** (JC159) DAKO	**CD34**	**CD117**	**CD13** (WM15) BD	**CD71** (M-A712) BD	**HLA-DR**	**CD45**

Per antibody CD number, (clone), and manufacturer are depicted. DAKO: DakoCytomation, Glostrup, Denmark; BD: BD Biosciences, San Jose, CA, USA. BL: BioLegend, San Diego, Ca, USA. Sanquin, Amsterdam, The Netherlands. BC: Beckman Coulter, Miami, FL, USA. IS: Immunostep, Salamanca, Spain.

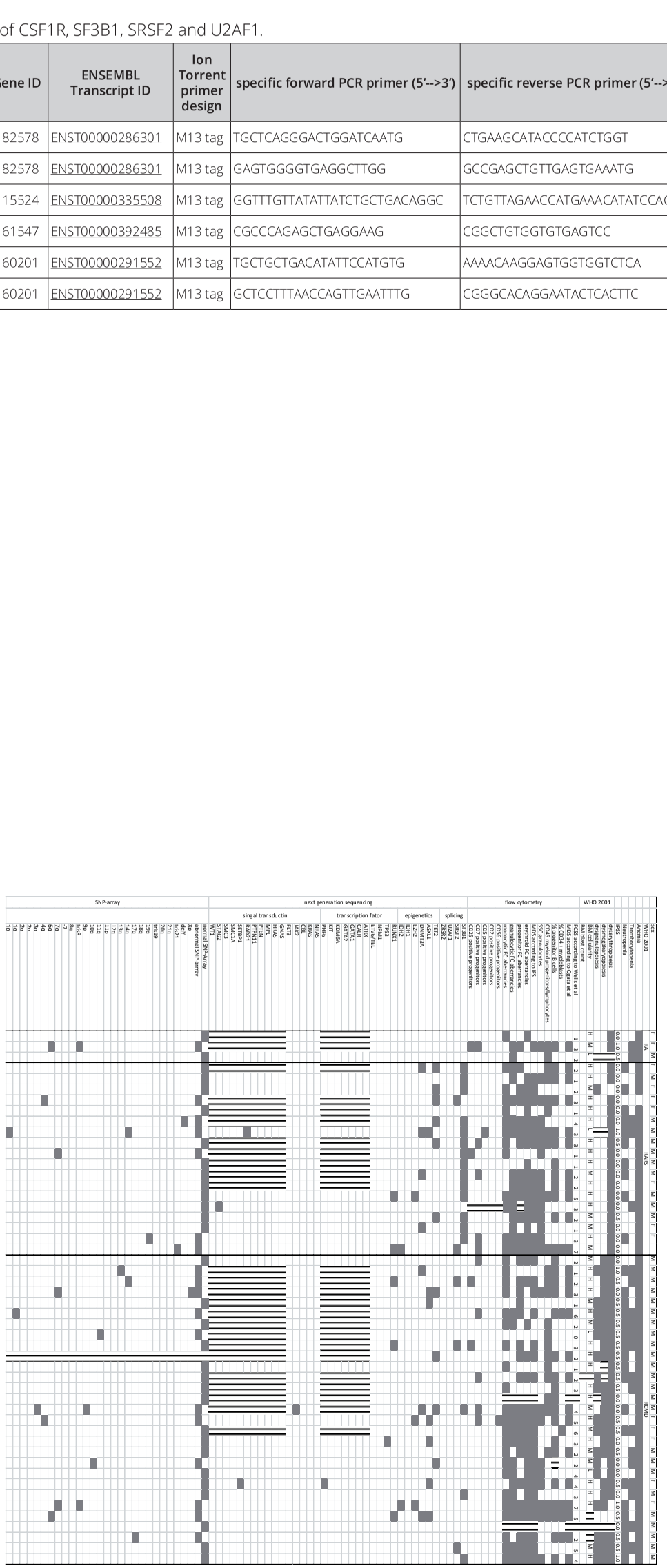
Table S2 The applied next-generation sequence panel.

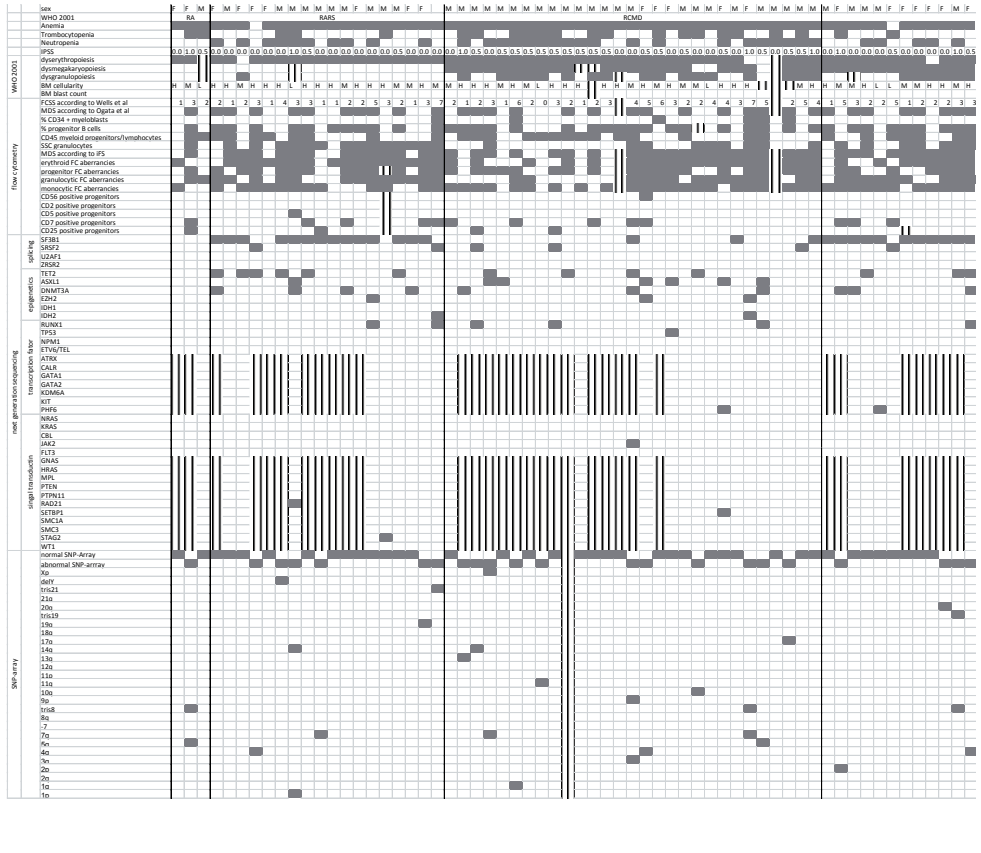
Table X: Composition of sequencing panel			
	Gene	**Genic regions covered**	**Gene function**
1	*ASXL1*	E13	Epigenetic regulation
2	*CBL*	E8, E9	Signal transduction
3	*CSF1R*	E7, E22	Growth factor receptor
4	*DNMT3A*	all coding exons (E2-E23)	Epigenetic regulation
5	*ETV6*	all coding exons (E1-E8)	Transcription factor
6	*EZH2*	all coding exons (E2-E20)	Epigenetic regulation
7	*FLT3*	E14-E15, E20	Growth factor receptor
8	*IDH1*	E4	Epigenetic regulation
9	*IDH2*	E4	Epigenetic regulation
10	*JAK2*	E12, E14	Signal transduction
11	*KRAS*	E2, E3	Signal transduction
12	*NPM1*	E11	Nuclear import, apoptosis
13	*NRAS*	E2, E3	Signal transduction
14	*RUNX1*	all coding exons (E1-E6)	Transcription factor
15	*SF3B1*	E6, E8, E13-E16	Splicing factor
16	*SRSF2*	E1	Splicing factor
17	*TET2*	all coding exons (E3-E11)	Epigenetic regulation
18	*TP53*	all coding exons (E2-E11)	Transcription factor, apoptosis
19	*U2AF1*	E2	Splicing factor
20	*ZRSR2*	all coding exons (E1-E11)	Splicing factor

Table S3 Amplicons covering relevant regions of CSF1R, SF3B1, SRSF2 and U2AF1.

Gene + Region name	Genomic coordinates (GRCh37.68)	ENSEMBL Gene ID	ENSEMBL Transcript ID	Ion Torrent primer design	specific forward PCR primer (5'-->3')	specific reverse PCR primer (5'-->3')
CSF1R_07	chr5, 149452806..149453153	ENSG00000182578	ENST00000286301	M13 tag	TGCTCAGGGACTGGATCAATG	CTGAAGCATACCCCATCTGGT
CSF1R_22	chr5, 149433512..149433882	ENSG00000182578	ENST00000286301	M13 tag	GAGTGGGGTGAGGCTTGG	GCCGAGCTGTTGAGTGAAATG
SF3B1_15-16	chr2, 198266410..198267032	ENSG00000115524	ENST00000335508	M13 tag	GGTTTGTTATATTATCTGCTGACAGGC	TCTGTTAGAACCATGAAACATATCCAG
SRSF2_01	chr17, 74732937..74733301	ENSG00000161547	ENST00000392485	M13 tag	CGCCCAGAGCTGAGGAAG	CGGCTGTGGTGTGAGTCC
U2AF1_02	chr21, 44524222..44524581	ENSG00000160201	ENST00000291552	M13 tag	TGCTGCTGACATATTCCATGTG	AAAACAAGGAGTGGTGGTCTCA
U2AF1_06	chr21, 44514562..44515125	ENSG00000160201	ENST00000291552	M13 tag	GCTCCTTTAACCAGTTGAATTTG	CGGGCACAGGAATACTCACTTC

Figure S1 the diagnostic landscape in low risk MDS.




sex
WHO 2001
Anemia
Thrombocytopenia
Neutropenia
IPSS
dyserythropoiesis
dysmegakaryopoiesis
dysgranulopoiesis
BM cellularity
BM blast count
FCSS according to Wells et al
MDS according to Ogata et al
% CD34 + myeloblasts
% progenitor B cells
CD45 myeloid progenitors/lymphocytes
SSC granulocytes
MDS according to IPS
erythroid FC aberrancies
progenitor FC aberrancies
granulocytic FC aberrancies
monocytic FC aberrancies
CD56 positive progenitors
CD2 positive progenitors
CD5 positive progenitors
CD7 positive progenitors
CD25 positive progenitors
SF3B1
SRSF2
U2AF1
ZRSR2
TET2
ASXL1
DNMT3A
EZH2
IDH1
IDH2
RUNX1
TP53
NPM1
ETV6/TEL
ATRX
CALR
GATA1
GATA2
KDM6A
KIT
PHF6
NRAS
KRAS
CBL
JAK2
FLT3
GNAS
HRAS
MPL
PTEN
PTPN11
RAD21
SETBP1
SMC1A
SMC3
STAG2
WT1
normal SNP-Array
abnormal SNP-arrray
Xp
delY
tris21
21q
20q
tris19
19q
18q
17q
14q
13q
12q
11o
11q
10q
9o
tris8
8q
-7
7q
6o
4q
3o
2o
2q
1q
1p
RA
RARS
RCMD
WHO 2001
flow cytometry
splicing
epigenetics
transcription factor
next generation sequencing
signal transduction
SNP-array

CHAPTER 7

INTRODUCTION

NAMENS HOVON WERKGROEP MDS/AML

E.M.P. CREMERS*, L. DE SWART*, G. HULS, P. WIJERMANS,
B. LÖWENBERG, M. JONGEN-LAVRENCIC, G.E. DE GREEF, P. MUUS,
M. VAN MARWIJK KOOY, R. SCHAAFSMA, T. VAN MAANEN, W. DEENIK,
A. BEEKER, R.E. BROUWER, M. HOOGENDOORN, D.A. BREEMS,
H.G.P. RAAIJMAKERS, G.E. VERHOEF, H.C. SCHOUTEN, P. VON DEM BORNE,
J. KUBALL, B.J. BIEMOND, E. VELLENGA, G.J. OSSENKOPPELE,
T.J.M. DE WITTE, A.A. VAN DE LOOSDRECHT.

NEDERLANDS TIJDSCHRIFT VOOR HEMATOLOGIE 2015; 5:215-221 (ADAPTED)

Transfusion dependency in MDS

About 80% of the MDS patients will present with an anemia, of which 40% will be treated with supportive care. This means erythropoietin and granulocyte-colony stimulating factor with or without red blood cell transfusions.[1,2] Red blood cell transfusions decrease anemia-related symptoms and prevent ischemic organ damage. However, every unit of red blood cells contains about 200-250 mg of iron, 100 times the normal daily amount. There is no physiological mechanism to erase this overload of iron from the body. This means that multiple blood cell transfusions might lead to a secondary hemochromatosis.

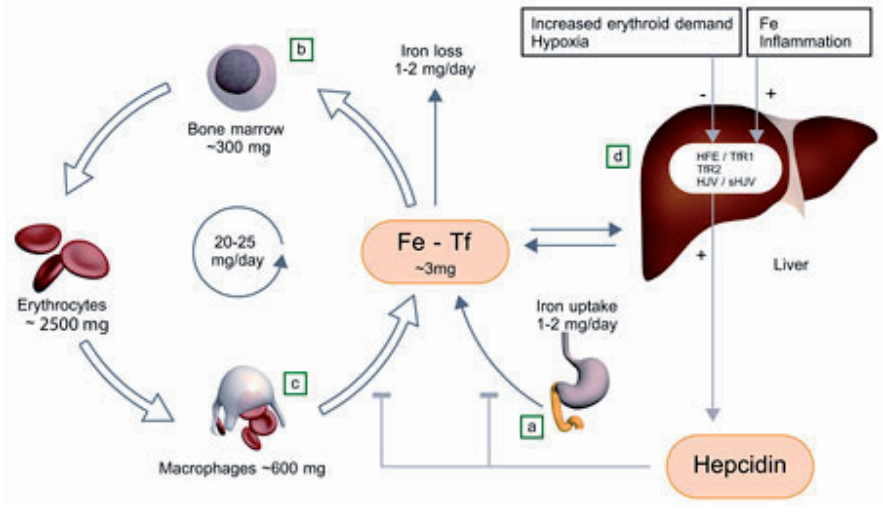
The only potential curative treatment for MDS is allogeneic stem cell transplantation. Here, high transfusion dependency predicts a poor treatment outcome.[3] If this is caused by the deleterious effect of iron, or that transfusions are only a reflection of the depth of the bone marrow disease, is unknown.

Pathophysiology of secondary hemochromatosis

The main regulator of iron homeostasis is hepcidin, a peptide hormone produced by the liver. Hepcidin breaks down the ferroportin, a membrane-bound iron transporter, through which iron is normally capable to leave the enterocytes and macrophages.

In case of an iron deficiency the hepcidin level in the blood is low, this leads to an increased iron absorption in the gut via the DMT1 transporter (Fe^{2+}) and HCP1 transporter (heme). Subsequently, iron reaches the circulation via ferroportin (Figure 1). A low hepcidin level will also cause release of stored iron from the macrophages of the reticulo-endothelial system (stored after erythrocyte degradation; under normal circumstances 20mg/day).[1]

Figure 1 Iron homeostasis.



In patients with MDS-RARS the erythropoiesis is increased but ineffective. In MDS-RARS patients hepcidin blood levels are significantly reduced. This is probably caused by release of humoral factors during the ineffective erythropoiesis, which suppresses the production of hepcidin. In MDS-RAEB patients an increased hepcidin blood level is found, mainly as a result of many red blood cell transfusions which lead to an increased iron in the circulation.[4,5] Each transfused unit of erythrocytes provides 200-250 mg of iron directly to the macrophages. The body has no active mechanism to remove excess of iron, but can passively remove 1-2 mg of iron per day through feces and sweat. Multiple transfusions will therefore lead to iron accumulation.

Iron is transported bound to transferrin. A small part is bound to ferritin and heme. The physiological storage possibilities are binding to ferritin in epithelial cells and macrophages. When the binding capacity of ferritin and transferrin is exceeded, iron occurs in the blood as 'non-transferrin bound iron' (NTBI). This means that the iron binds to other circulating molecules such as citrate, acetate and albumin. These bindings are less stable than the bindings to transferrin, ferritin or heme. The most reactive form of NTBI is called labile plasma iron (LPI). LPI easily passes the cell membrane and initiates cell damage in organs due to an increase in reactive oxygen species (ROS; oxygen free radicals) via the Fenton reaction (breakdown of H_2O_2 by reaction with an iron (II) ion). Oxidative stress and ROS-induced damage is a potential trigger for the increased apoptosis of the erythroid precursors in MDS by increasing of the Fas receptor (and the corresponding ligand FasL). This has a possible negative influence on survival. However, more research is needed to explain the direct toxic effects of red blood cell transfusions.[6,7]

Clinical effects of secondary hemochromatosis

As mentioned, the iron status becomes misbalanced after each transfused unit of red blood cells.[8] As a result, secondary hemochromatosis will arise in patients who have received multiple red blood cell transfusions. The described negative effect of transfusion dependence on survival can be explained in two ways: i) as a reflection of the degree of bone marrow failure; ii) as a result of iron toxicity caused by transfusions. Bone marrow failure may lead to a higher risk of infection, hemorrhages and the negative effects of a prolonged-existing anemia, such as reduced quality of life, the risk of heart failure, and tissue ischemia (Figure 1). Studies on long-term transfusion-dependent patients with thalassemia revealed that heart, liver and endocrine organs are the most commonly affected organs by iron overload. Correlations between these complications and transfusion-caused iron overload are difficult to interpret as i) MDS is a very heterogeneous disease; ii) in general, MDS patients are elderly persons, usually with age-related comorbidities; iii) the mortality rate is heavily influenced by age. This makes it difficult to correlate

morbidity and mortality, solely with transfusion dependency. Described transfusion-related complications in MDS are: liver cirrhosis by iron accumulation in the liver, diabetes mellitus due to iron overload in the pancreas, vascular damage caused by direct toxic damage of free iron in the blood and cardiac failure as a result of iron overload in the hart, and reduced quality of life.[6,9] However, it remains complex to rule out other causes such as high age, pre-existing cardiovascular comorbidity and/or underlying chronic anemia due to chronic illnesses, especially in vascular diseases or cardiac failure.

Influence on (leukemic-free) survival

In untreated patients with very low and low risk MDS, the median survival is 8.8 (IPSS-R). This means that in transfusion-dependent patients, iron accumulation takes place over a long period. A prospective analysis within the European LeukemiaNet MDS-registry showed progression to AML in 5% of the low- and intermediate-1-risk (IPSS) MDS patients within 5-years; 12% died due to another cause.[10] The most common causes of death in this group were infections and cardiovascular complications. Transfusion-dependent patients without disease progression had a four-times higher risk of death within 2 years, when compared to transfusion-independent patients. In contrast, there was no difference in survival between patients with disease progression, with or without transfusion dependency.

Patients with high-risk MDS are expected to show fast progression to AML. So far, the only potentially curative therapy for high-risk patients is allogeneic stem cell transplantation (allo-SCT). Retrospective studies in this patient population showed that patients with high ferritin levels before the transplantation have a higher transplant-related mortality than patients with low-to-normal ferritin levels.[11,12] However, a recent prospective evaluation showed that iron overload, measured by T2-weighted MRI, had no impact on treatment-related mortality or overall survival after allo-SCT.[13] Although there are indications that transfusion dependency and associated iron overload have a negative effect on allo-SCT outcome, clear evidence for a positive effect of iron-reducing therapy (via chelation drugs or phlebotomies) in a transplantation setting is lacking. However, it is advised to measure the degree of iron accumulation in allogeneic SCT candidates.[14]

Aim of the thesis – part 2

The aims of the second part of the thesis are to investigate the deleterious effect of transfusion-related iron toxicity in patients with high risk MDS treated with allo-SCT and to investigate a rationale for iron chelation therapy in an allo-SCT setting.
Chapter 9 and **chapter 10** describe results of clinical trials conducted within the Chronic Malignancies Working Party (CMPW) of the European Society for Blood and Marrow Transplantation (EBMT). **Chapter 9** describes the retrospective investigation of prognostic factors in MDS patients who were treated upfront with an allo-SCT. **Chapter 10** describes a prospective analysis of the influence of transfusion dependency on treatment outcome in MDS patients also treated upfront with allo-SCT.

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CHAPTER 8

PROGNOSTIC PRE-TRANSPLANT FACTORS IN MYELODYSPLASTIC SYNDROMES PRIMARILY TREATED BY HIGH DOSE ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

A RETROSPECTIVE STUDY ON BEHALF OF THE MDS SUBCOMMITTEE OF THE CHRONIC MALIGNANCIES WORKING PARTY OF THE EUROPEAN BLOOD AND MARROW TRANSPLANTATION ORGANIZATION

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Abstract

Many pre-transplant factors are known to influence the outcome of allogeneic stem cell transplantation (SCT) treatment in myelodysplastic syndromes (MDS). However, patient cohorts are often heterogeneous by disease stage and treatment modalities, which complicates interpretation of the results. This study aimed to obtain a homogeneous patient cohort by including only de novo MDS patients who received upfront allogeneic SCT after standard high dose myeloablative conditioning. The effect of pre-transplant factors such as age, disease stage, transfusions, iron parameters and comorbidity on overall survival (OS), non-relapse mortality (NRM) and relapse incidence (RI) was evaluated in 201 patients. In this cohort, characterized by low comorbidity and a short interval between diagnosis and transplantation, NRM was the most determinant factor for survival after SCT (47% after 2-year follow-up). WHO-classification and transfusion burden were the only modalities with a significant impact on overall survival after SCT. Estimated Hazard Ratios (HR) showed a strongly increased risk of death, NRM and RI, in patients with a high transfusion-burden (HR 1.99; P=0.006, HR of 1.89; P=0.03 and HR 2.67; P=0.03). The HR's for ferritin level and comorbidity were not significantly increased.

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of myeloid bone marrow disorders characterized by clonal hematopoiesis, impaired differentiation, peripheral cytopenias, and an increased risk of progression to acute myeloid leukemia (AML).[1] Allogeneic hematopoietic stem cell transplantation (SCT) is considered the only modality with proven curative potential, but leads to considerable treatment-related morbidity, mortality and decreased quality of life.[2-5]

Most MDS patients receive red blood cell (RBC) transfusions during the course of their disease.[6] Transfused patients are prone to long-term accumulation of iron due to red blood cell (RBC) transfusions as well as susceptible to direct iron toxicity due to the formation of reactive oxygen species (ROS).[7] Iron accumulation in MDS may start before patients become transfusion-dependent because of ineffective erythropoiesis, which blocks hepcidin production and subsequently increases iron absorption from the gut. Myelosuppressive therapy blocks erythropoiesis immediately and may result in direct iron toxicity.[8] Elevated toxic iron radicals may lead to an increased risk of infections, higher SCT mortality, leukemic transformation, and tissue damage.[9-11] Many studies described the negative impact of transfusion-dependency and associated iron accumulation on treatment outcome after SCT. Serum ferritin has often been used as a surrogate marker for iron overload. It is associated with an adverse effect on overall survival (OS), non-relapse mortality (NRM) and relapse incidence (RI).[4, 12-15] However, ferritin levels are also associated with advanced stages of MDS, number of prior regimens and infections.[16] Therefore, pre-transplant ferritin levels are less suited to evaluate iron toxicity caused by transfusions and ineffective erythropoiesis in MDS.

High dose chemotherapy decreases erythropoiesis and utilisation of iron, which leads to an increased iron load.[17] High dose myeloablative preparative regimens cause more serious organ toxicity, higher risk of infections and acute graft-versus-host disease (GvHD) than non-myeloablative regimens.[18] Therefore, this study aimed to analyse a homogeneous group of MDS patients only treated with high dose chemotherapy as part of the transplant conditioning, without previous anti-leukemic treatment (to minimize comorbidity). This allows more insight in the role of transfusion-burden and associated iron accumulation during SCT procedures. Insight in factors contributing to NRM may lead to better treatment approaches in patients who are to be treated with SCT.

Patients and Methods

The Chronic Malignancies Working Party (CMWP) of the European Group of Blood and Marrow Transplantation (EBMT) collected retrospective data of adult patients with proven MDS according to the WHO-classification, who received allogeneic SCT after high dose conditioning. Centers who had transplanted more than 4 MDS patients between 2000 and 2005 were invited to participate in this survey. Due to strict inclusion criteria 34 centers were selected to participate; leading to a cohort of 243 patients (range: 1 to 19 per center). The data were checked on diagnosis, primary origin, and previous treatment with intensive chemotherapy. With this

Table 1. Baseline characteristics at time of transplantation of all patients in the study

	Total (n=201)	≤20 RBC units (n=86)	>20 RBC units (n=41)	P-value*
Median age (range) (n=201)	49 (18-70)			
≤ 50 years	103 (51%)	43 (50%)	17 (41%)	0.37
> 50 years	98 (49%)	43 (50%)	24 (59%)	
Sex (male)	119 (59%)	52 (61%)	24 (59%)	0.84
WHOclassification (n=201)				
RA/RARS/5q-	72 (36%)	26 (30%)	14 (34%)	0.90 **
RCMD	15 (8%)	3 (4%)	2 (5%)	
RAEB-1	34 (17%)	20 (23%)	5 (12%)	
RAEB-2	39 (19%)	18 (21%)	11 (27%)	
MDS/AML	41 (20%)	19 (22%)	9 (22%)	
Cytogenetics (n=152)***				
Good	67 (44%)	32 (41%)	17 (47%)	0.64
Intermediate	43 (28%)	24 (31%)	8 (22%)	
Poor	42 (28%)	22 (28%)	11 (31%)	
Median time Dx-Tx	8 (0.3-274)			
≤6 months	80 (40%)	40 (47%)	11 (27%)	0.03
>6 months	121 (60%)	46 (53%)	30 (73%)	
Donor type (n=199)				
Sibling	110 (55%)	42 (49%)	18 (45%)	0.65
Unrelated donor	89 (45%)	43 (51%)	22 (55%)	
Match sex recipients-donor (n=201)				
Male-female	46 (23%)	19 (22%)	8 (20%)	0.74
Other	155 (77%)	67 (78%)	33 (80%)	
Comorbidity (n=145)				
No	95 (66%)	54 (63%)	28 (68%)	0.54
Yes	50 (34%)	32 (37%)	13 (32%)	

Univariate comparisons between baseline characteristics of patients who received ≤20 RBC units and those who received >20 RBC units. (*) P-values are derived from the Chi square test or Cochran-Armitage test for trend (**). According to IPSS risk categories (***).

primary origin, and previous treatment with intensive chemotherapy. With this procedure 24 patients were excluded because of secondary origin, 18 patients because of missing relevant transplantation data. The additional survey collected data which are not routinely recorded in the EBMT registry, including ferritin levels, serum iron levels, transferrin saturation and number of RBC-transfusions. Patients' health status, including co-morbidities, was recorded through follow-up forms up to 5 years post-transplant. All clinical variables were measured at time of transplantation in patients undergoing upfront SCT, without other pre-transplant anti-leukemic treatment. Patients were analyzed according to patient and donor characteristics, WHO-classification, number of RBC units transfused, presence of comorbidity, iron parameters including ferritin, transferrin and plasma iron, and cytogenetic risk category according to the International Prognostic Scoring System (IPSS) risk categories.[5, 19] Patients were diagnosed and classified prior to the introduction of the revised IPSS, therefore the IPSS was applied. Data on extra-hematologic comorbidities, that can influence the outcome of treatment were calculated using the Sorror co-morbidity index.[18] The procedures were in accordance with the ethical standards with the Declaration of Helsinki.

End points and statistical analysis

Primary end-points were OS, NRM, and RI. OS was defined as the probability of survival since transplantation; death from any cause was considered as an event. Patients alive at time of last follow-up were censored at this date. NRM was defined as the probability of any death in the absence of relapse since SCT. RI was defined as the probability of hematologic relapse (definition: cytological and/or histological evidence of the disease in the marrow-blood and/or in extramedullary sites after SCT). For NRM and RI, patients were censored if relapse free and alive at time of last follow-up.

The probabilities of OS were estimated using the Kaplan-Meier product limit method. Estimates of NRM and RI were calculated using cumulative incidence curves to accommodate competing risks (relapse, considered a competing risk for NRM and vice versa). Univariate comparisons were based on the Kaplan-Meier method for OS, and on non-parametric cumulative incidence curves for RI and NRM. All significance tests are Cox-model based score tests (corresponding to the usual log-rank tests for OS and NRM).

Cox proportional hazards regression was used to assess the impact of potential prognostic factors in multivariate analyses. The impact of these factors on OS, NRM and RI was modeled by means of cause-specific hazards. For each outcome, we created 3 models. The first model contained baseline (expected) predictive factors: WHO-classification, age, donor type, sex-match (a female donor-male recipient combination has been described as a negative influence on SCT outcome)[20], time

between diagnosis and SCT and cytogenetic abnormalities (model 1; see table 2).[6, 21] Then we added RBC-transfusions, and comorbidity, respectively (models 2-3). We checked the impact of missing values for the key variables RBC-transfusions and comorbidity score on the outcomes both in the univariate and in the multivariate analyses. Since we concluded that the estimates of the coefficients of interest were not influenced significantly by the presence or absence of the patients with missing values for each of these key variables in turn, we presented the results based on different subsets of our data set with non-missing information.

Analyses were performed using PASW Statistics version 18.0 (SPSS, Chicago, IL). Cumulative incidences were calculated by means of SPSS macros developed by the Department of Medical Statistics and Bioinformatics of the Leiden University Medical Center (Leiden, the Netherlands); they are based on the hazard estimates from the Cox models. All P-values are two-sided and P<0.05 was considered significant. The dataset for analysis was closed in March 2011.

Results

A total of 201 patients underwent allogeneic SCT after high dose conditioning for untreated MDS between 2001-2005. Table 1 describes patients' characteristics. The median age was 49 years (range 18-70) and 119 patients were male.

Due to the selection criteria of upfront SCT without prior treatment, the group of patients with refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS) or isolated deletion of the 5q chromosome (5q-) population was relatively large (36%) in comparison to other reports [1]. It is important to realize that this study was conducted prior to the introduction of lenalidomide and hypo-methylating agents. Treatment regiments were high dose myeloablative regimens, most commonly based on Busulfan (N=82), Treosulfan (N=15) and Melfalan (N=15) or total body irradiation (TBI; N=62) in dosages used in myelo-ablative schedules.[22, 23] GvHD prophylaxis regimens comprised of Cyclosporin (N=159), methotrexate (N=107), mycophenolate mofetil (N=24) and/or ATG (N=65). Median time between diagnosis and SCT was 8 months. Pre-SCT comorbidity was present in 34% of the patients. Of the patients transfused, 23% received >20 RBC units. Mean pre-transplant iron parameters were: ferritin 1288ng/mL (N=62; range 72-9695), transferrin 233 mg/dL (N=30; range 56-538), transferrin saturation 29% (N=23; range 7-160), serum iron 171 mg/dL (N=43; range 30-467). Six patients received iron chelation post-SCT. The Sorror comorbidity index (HCT-CI) subdivided patients in 'no comorbidity' (HCT-CI: 0) 66%, 'mild/moderate comorbidity' (HCT-CI of 1-2) 25%, or 'severe comorbidity' (HCT-CI of ≥3) 9%.

The OS, NRM and RI at 2 years post-SCT were 47%, 41% and 14%, respectively. Figure 1 illustrates the OS stratified for WHO-classification, RBC-transfusions, ferritin level, and comorbidity. In univariate analyses, WHO-classification significantly affected OS (P=0.04) and RI (P=0.003), but did not affect NRM (P=0.38). Age, cytogenetics, donor type, match sex recipient-donor, and time between diagnosis and SCT did not have a significant impact on OS, NRM and RI in univariate analyses (data not shown).

Treatment outcome according to transfusion-burden

To examine the influence of transfusion-burden, patients were subdivided into 2 groups depending on the amount of RBC units received pre-SCT, ≤20 and >20 RBC-transfusions. Patients with a low transfusion burden had a significantly (P = 0.006) higher 2-year OS than patients with a high transfusion-burden (52% versus 31%), which was mainly explained by a higher NRM for patients with >20 RBC transfusions. The 2-year NRM was different in both groups: 54% for patients with >20 RBC-transfusions, compared to 36% for the patients with less RBC-transfusions (P=0.02). The RI was comparable in both groups: 17% and 15% at 2 years (P=0.20).

Treatment outcome according to ferritin levels

Ferritin levels prior to transplantation were reported in a minority of patients (N=62). Based on ferritin levels patients were subdivided into two categories: <1000ng/ml (N=35) and ≥1000ng/ml (N=27). Patients with a higher ferritin level had a 14% lower 2-year survival than patients with a normal ferritin level (37% versus 51%; Figure 1) (P=0.23). The 2-year NRM and RI were 44% and 19%, in patients with a ferritin level ≥1000ng/ml, compared to 43% and 9%, in patients with a ferritin level below 1000ng/ml. The differences between the RI of both groups were not significant.

Treatment outcome according to comorbidity

Since the number of patients with co-morbidities was too small to subdivide the patients according to Sorror comorbidity index, presence and absence of comorbidities was used. The Kaplan Meier curves showed a 10% decrease in 2-year survival for the patients with comorbidity (40% versus 50%). However, the difference in OS was not significant (P=0.15). RI was equal in both groups (16% vs 14%; P=0.63). NRM for patients with a comorbidity was 48%, compared to 37% for patients without comorbidity (P=0.12).

Multivariate analysis of pre-transplant factors

Cox models were built for the multivariate analysis (see patients and methods). The baseline model, which included expected prognostic factors showed that transplantation with an unrelated donor had a very significant impact on the HR for RI (HR 0.28; P=0.002) but no influence on survival (HR 1.03; P=0.89) (table 2). Although other studies used 40 years of age as a cut-off point, we decided to use 50 years since this was closer to the median.[2] Poor risk cytogenetic abnormalities (≥3 abnormalities or an abnormal chromosome 7) had a significant impact on the HR for RI (HR 3.01; P=0.009). This did not influence survival significantly (HR 1.31; P=0.29). WHO-classification influenced the outcome significantly (table 2). In comparison to RA/RARS patients, patients with a refractory cytopenia with multilineage dysplasia (RCMD) had a statistically decreased survival (HR 3.12; P=0.001) and high HR for NRM (HR 2.80; P=0.006). This impact remained significantly increased in all models. In comparison to RA/RARS patients, patients with a refractory anemia with excess blast-1 (RAEB-1) and patients with MDS transformed into AML (MDS/AML) had a significantly higher risk of relapse, HR 5.95 (P=0.001), and HR 5.66 (P=0.003), respectively, leading only to a significantly decreased survival in MDS/AML patients (HR1.85; P=0.03).

Age, sex match donor-recipient, and interval between diagnosis and transplantation had no significant prognostic impact on OS, NRM and RI in the baseline model (table 2).

The Cox models built for transfusion-burden showed that a high transfusion-burden resulted in an inferior outcome for OS, NRM and RI, with a HR of 1.99 (P=0.006), 1.89 (P=0.03), and 2.67 (P=0.03) respectively (model 2). The presence of comorbidity was associated with a non-significant increased risk of death (1.43; P=0.15) and NRM (HR 1.67; P=0.08) but no impact on RI (HR 0.71; P=0.44) (table 2; model 3).

Discussion

Several studies addressed the impact of transfusion-dependency, transfusion-burden and iron overload/toxicity after transplantation in MDS patients. However, data from these retrospective studies should be interpreted carefully because patient cohorts are often heterogeneous with regards to disease status, comorbidity, and treatment modalities.[19, 24] High risk patients frequently receive cytoreductive therapy (intensive chemotherapy or hypomethylating agents) prior to the transplant conditioning, sometimes as part of bridging. The value of cytoreductive strategies are not supported by retrospective and prospective studies.[25]

Table 2 Multivariate analysis for Overall Survival, Non-relapse Mortality and Relapse incidence with traditional patient, disease and transplantation related variables (model 1) extended with RBC-transfusions (model 2), and comorbidity (model 3), respectively.

	Overall Survival		Non-Relapse Mortality		Relapse Incidence	
	HR	p-value	HR	p-value	HR	p-value
Model 1 (n=199)						
Donor type (HLA matched unrelated vs sibling)*	1.03	0.89	1.20	0.43	**0.28**	**0.002**
*Cytogenetic abnormalities***		0.54		0.91		0.02
Good and intermediate	1		1		1	
Poor	1.31	0.29	0.98	0.94	**3.01**	**0.009**
Time Dx-Tx (>6 months vs ≤6 months)	1.31	0.20	1.33	0.24	1.26	0.54
WHO classification		0.01		.03		0.006
RA/RARS	1		1		1	
RCMD	**3.12**	**0.001**	**2.80**	**0.006**	3.58	0.15
RAEB-1	1.15	0.65	0.77	0.48	**5.95**	**0.001**
RAEB-2	1.57	0.13	1.41	0.29	1.81	0.35
MDS/AML	**1.85**	**0.03**	1.40	0.28	**5.66**	**0.003**
Age (≤50 vs >50 years)	1.28	0.24	1.12	0.64	1.85	0.10
Sex-match donor-recipient (m-f vs other)	1.05	0.84	0.99	0.98	1.11	0.80
Model 2 (n=125)						
RBC-transfusion (>20 RBS-units vs ≤20 RBC-units)	**1.99**	**0.006**	**1.89**	**0.027**	**2.67**	**0.031**
Model 3 (n=143)						
Comorbidity (yes vs no)	1.43	0.15	1.67	0.08	0.71	0.44

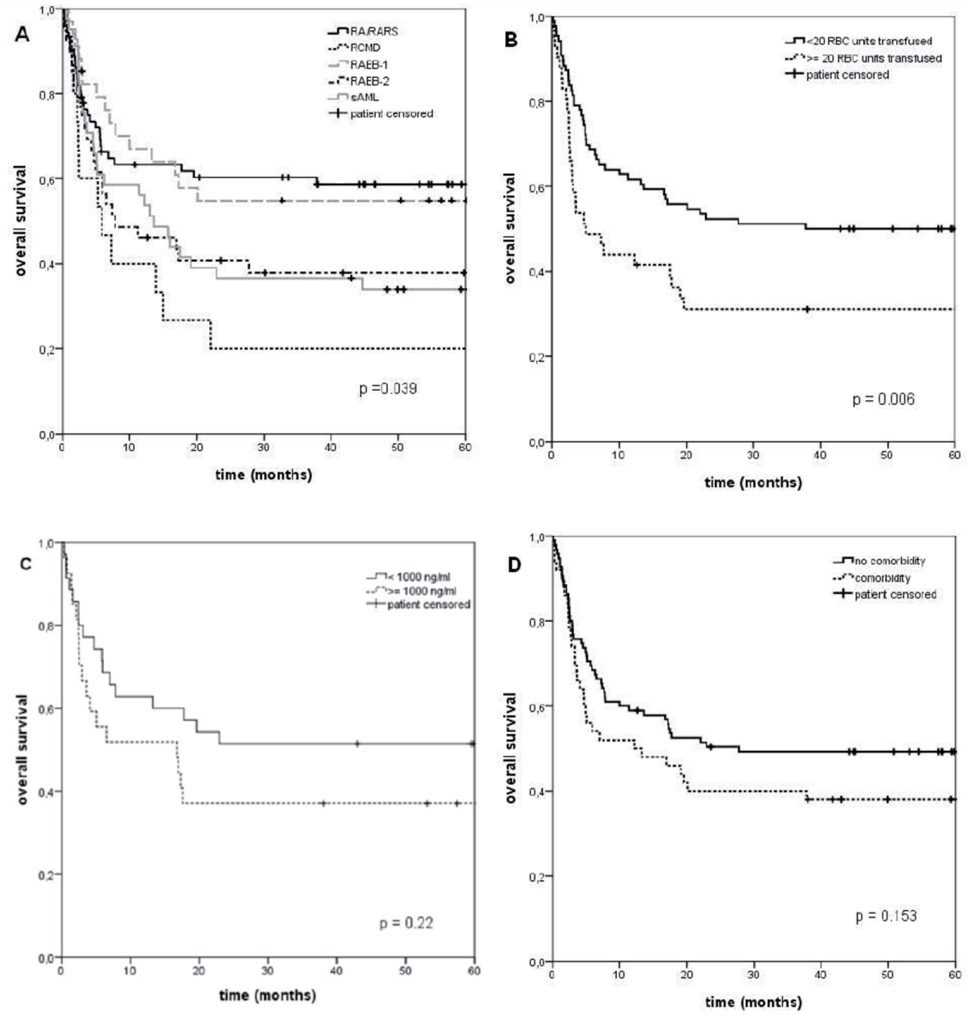
Cox regression models for the (cause-specific) hazards were fitted on different restricted datasets with full information on the main variables of interest. The entries in the table are Hazard Ratios and their associated p-values and group size. HRs (with their p-values) in bold have a statistically significant impact on OS, NRM or RI, at the 5%-level. (*) Mismatched siblings and mismatched unrelated donors are included in the HLA matched unrelated donor category. (**) patients with missing information for this variable were kept in the analysis with variable level 'missing' (HRs not shown).

We have chosen this this homogeneous population with a relative low relapse risk after transplantation and a long median follow-up of 5 years. Both in AML and in MDS, ferritin levels had a pronounced influence on OS in heavily pretreated patients.[12, 16] Since ferritin is an acute phase reactant, we postulate that elevated ferritin levels are heavily influenced by stage of disease and by recently applied intensive chemotherapy and associated invasive infections. Therefore, the homogeneity of this cohort may provide more insight in the impact of relevant prognostic factors in the setting of SCT. In addition, we focused on obtaining a complete pre-transplantation transfusion history to be able to accurately analyze the impact of transfusion burden rather than using ferritin levels as surrogate marker.

In this study NRM was the most determinant factor for survival post-SCT, probably due to the large number of patients with <5% blasts (36%). The favorable OS of the large RA/RARS group may explain the lack of prognostic impact of several putative prognostic factors, including age, sex-match donor-recipient, time between diagnosis and SCT and cytogenetic abnormalities.[19] Cytogenetic abnormalities did have an impact on RI, but not on OS since NRM was the driving force of death in this study. In all models age had no impact on outcome. The low incidence of comorbidities may explain the loss of the prognostic impact by age in this patient cohort. As expected WHO-classification had a major impact on outcome (P=0.04). In comparison to RA/RARS patients, RCMD patients showed a significantly decreased survival (HR 3.12; P=0.001). The HR's of RCMD for OS and NRM remained significantly increased after adding transfusion-burden, and comorbidities to the models. The increase of the HR after adding number of transfusions and co-morbidity suggests that the impaired survival is intrinsic to this MDS category. However, this unique observation needs confirmation by additional studies. As expected, RAEB-1 and MDS/AML patients had a significantly higher risk on RI, 5.95; P=0.001, and HR 5.66; P=0.003, compared to RA/RARS patients, leading to a significantly decreased survival in MDS/AML patients (HR1.85; P=0.03).

Transfusion dependency appeared to have a major prognostic impact on outcome. Multivariate analysis showed a significantly decreased OS in patients who received >20 RBC-transfusions prior to conditioning (HR 1.99; P=0.006), due to an increased NRM and RI with a HR of 1.89 (P=0.03) and HR 2.67 (P=0.03) respectively. A higher transfusion-dependency may indicate a more pronounced marrow failure which may be associated with a decreased survival. In addition toxicity caused by RBC-transfusions might be deleterious either because of increasing iron load or because of other adverse effects e.g. transfusion related immunomodulation (TRIM) and the effect of stored blood on the microcirculation hemodynamics and

Fig 1. Overall survival stratified for WHO-classification (A), transfusion burden (B), iron load (C), and comorbidity (D) (Kaplan-Meier curves). P-values are based on the two-sided log-rank test.



tissue oxygenation.[26, 27] A more challenging explanation is given by Hod et al.[28] They described that RBC-transfusions with stored blood give a sudden rise in non-transferrin bound iron (NTBI), due to a rapid clearance of the damaged blood cells. This sudden increase in NTBI may enhance transfusion-related complications.[9, 10] To minimize transfusions toxicity they advised to use fresh erythrocytes for transfusions, which will have a major impact on the logistics of clinical practice. Iron chelating therapy before and after SCT might be a good alternative. Chelation

therapy may improve hemoglobin levels and reduce transfusion requirement in a minority of patients with MDS.[29] Reduction of the interval between diagnosis and transplantation minimizes the exposure time to ineffective erythropoiesis, transfusion-dependent period and the number of transfusions. Comorbidity in this studied population was limited, which may explain the absent prognostic impact in contrast to other studies.[3] A prospective non-interventional study within the CMWP of the EBMT, to look at the influence of iron toxicity and transfusions on treatment outcome after allogeneic SCT in MDS patients has been completed and the analysis of the data is ongoing.

In summary, in this homogeneous patient cohort, NRM was the most determinant factor for survival after SCT. WHO-classification and transfusion-burden were the only pre-transplant factors with a significant impact on survival. Cytogenetic abnormalities had only a significant influence on the HRs for RI. More research on the influence and pathophysiology of transfusion toxicity is mandatory in particular the role of iron chelation before SCT and phlebotomies and/or iron chelation after SCT.

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CHAPTER 9

A PROSPECTIVE NON-INTERVENTIONAL STUDY ON THE IMPACT OF TRANSFUSION BURDEN AND RELATED IRON TOXICITY ON OUTCOME IN MYELODYSPLASTIC SYNDROMES UNDERGOING ALLOGENEIC HAEMATOPOIETIC CELL TRANSPLANTATION

A STUDY ON BEHALF OF THE MDS SUBCOMMITTEE OF THE CHRONIC MALIGNANCIES WORKING PARTY OF THE EUROPEAN SOCIETY FOR BLOOD AND MARROW TRANSPLANTATION

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SUBMITTED

Abstract

Most patients with myelodysplasic syndromes (MDS) present with anaemia and receive multiple red blood cell transfusions (RBCT). Transfusions may cause iron overload-related comorbidity and mortality, and may influence outcome after allogeneic HSCT. This prospective non-interventional study evaluated 222 patients with MDS and CMML who underwent upfront HSCT. Overall survival (OS), relapse-free survival (RFS), non-relapse mortality (NRM), and relapse incidence (RI) at 36 months were 52%, 44%, 25% and 31%, respectively. Age, percentage of marrow blasts and presence of severe comorbidities impacted OS (p=0.002, p=0.002, and p=0.02 respectively). RFS was significantly associated with RBCT burden prior to HSCT (HR 1.7; p=0.02) in the multivariable analysis. High ferritin levels had a significant negative impact on OS and RI, but no impact on NRM. Administration of iron chelation therapy prior to HSCT did not influence outcome, but iron reduction after HSCT either by phlebotomies or by iron chelation improved relapse-free survival significantly, if started before 6 months after transplantation.

This study illustrates the impact of RBCT and related parameters on outcome after transplantation. Patients with an expected prolonged survival after transplantation may benefit from early iron reduction therapy after transplantation.

Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) is the most potent curative therapeutic option in patients with myelodysplastic syndromes (MDS).[1] Due to high treatment-related morbidity and mortality, combined with a high relapse risk, the current long-term overall survival rate is around 35%.[2-4] Non-relapse mortality (NRM) has decreased after introduction of reduced intensity regimens (RIC) for the elderly and more frail patients with MDS.[5,6] Many parameters such as age, comorbidity, advanced disease stage and cytogenetic risk category according to the (revised)-International Prognostic Scoring system (IPSS-R) have been reported to impact treatment outcome.[4,7-10] Around 80% of patients with MDS present with anaemia and many of these patients receive multiple red blood cell transfusions (RBCT) during the course of their disease.[11] RBCT and associated iron overload may cause iron toxicity related comorbidity and mortality, and influence HSCT outcome.[12-15] Most studies are retrospective and selection bias and lack of detailed data about treatments, outcomes and potentially confounding risk factors might have influenced conclusions.

The Chronic Malignancies Working Party (CMWP) of the EBMT performed a large prospective, non-interventional study to evaluate prognostic pre-transplant factors in MDS and chronic myelomonocytic leukaemia (CMML). The primary objective of this study was to evaluate the relation between iron toxicity and treatment-related mortality after allogeneic HSCT in adult MDS and CMML patients. As previous intensive chemotherapy might have an impact on pre-HSCT co-variables, including stage of disease, patients treated with intensive chemotherapy were excluded from this study. This approach is expected to be associated with a reduced pre-treatment related morbidity, and a reduction of the interval between diagnosis and HSCT. Secondary objectives were i) description of treatment outcomes defined by NRM, OS, relapse incidence (RI) and relapse-free survival (RFS); ii) impact of RBCT and surrogate iron markers that may reflect iron burden, including ferritin levels, on treatment outcome. Data on iron reduction therapy prior and post HSCT was collected, to evaluate its impact on treatment outcome.

Patients and Methods

Two hundred twenty-two adult (age >=18 years at HSCT) patients with de-novo MDS, AML-MDS with 20 to 30% marrow blasts (formerly RAEBt), patients with AML after MDS (AML-MDS) or patients with CMML who received upfront HSCT after bone marrow ablative or reduced intensity regimens were included prospectively

between January 2009 and January 2014. Patients had an ECOG performance status between 0 and 2. Exclusion criteria included previous intensive anti-leukaemic chemotherapy, patients with juvenile CMML, patients with therapy-related MDS, AML or CMML after treatment with immunosuppressive or cytotoxic treatment for a non-myeloid malignancy, patients who had received auto-HSCT, candidates for cord blood HSCT or syngeneic HSCT, inadequate renal function (ECC <60 ml/min and/or creatinine >2.5 times upper limit of normal value), inadequate hepatic function (transaminases >2.5 times upper limit of normal value), history of seizures, pregnancy and women of child-bearing potential and not using adequate contraceptives, uncontrolled hypertension. The research was conducted according to the Declaration of Helsinki and written informed consent was obtained from each participating patient. Trial registered at Clinicaltrial.gov Identifier: 842205547.

Data were collected by survey at diagnosis, at transplantation and 6 weeks, 100 days, 1 year and annually thereafter. Missing data was collected by follow-up surveys. Based on the study objectives the following variables were collected: age, gender, comorbidities, WHO classification, cytogenetic characteristics according to IPSS-R, number of transfusions, ferritin levels, haemoglobin levels, C-reactive protein (CRP), donor type, female-donor/male recipient versus other combinations, interval diagnosis and HSCT, HSCT conditioning agents and types, T-cell depletion, acute and chronic graft versus host disease (GvHD), and data concerning treatment with iron chelation or phlebotomy.

Primary end-points were overall survival (OS), relapse-free survival (RFS), relapse incidence (RI), and non-relapse mortality (NRM), evaluated at 36 months after transplant. The median follow-up was estimated using the reverse Kaplan-Meier method. Additionally, acute GvHD grade I-II and III-IV and limited and extensive chronic GvHD were evaluated at 100 days and 36 months after transplant respectively. OS and RFS were estimated using the Kaplan-Meier product limit estimation method, and differences in subgroups were assessed by the Log-Rank test. Cumulative incidences of relapse and NRM, were analyzed in a competing risks framework. The cumulative incidences of acute GvHD grade I-II and III-IV were estimated as competing risks, considering as competing event death before aGvHD. The cumulative incidences of limited and extensive cGvHD were estimated equivalently. Subgroup differences were assessed by Gray's test. Cox proportional hazards regression was used to assess the impact of potential risk factors in univariable and multivariable analyses. Multivariable analyses of RI and NRM were performed using Cox cause-specific hazards models. The impact of post-transplant iron reduction therapy was investigated using landmark analyses

at 6, 12 and 24 months. The landmark population was defined as patients alive (event = death) and event free (event = death and/or relapse) at the respective landmark time points. All p-values were two-sided and p<0.05 was considered significant

Results

Demographic and transplantation data

The study included 181 patients with MDS, 16 patients with AML-MDS, and 25 patients with CMML from 29 European transplant centres (for details, see table 1). The median age was 59 years (range 19-76 years). WHO-classifications at time of transplantation were 20 patients with a RA/RARS, 36 with RCMD/RCMD-RS, 1 with 5q-, 50 with RAEB-1, 74 with RAEB-2, 25 with CMML and 16 with AML-MDS. In total, 70% of the patients had received red blood cell transfusions (RBCT) prior to HSCT. Median number of RBC units transfused before transplantation: 12 units (range: 1-146 units); median ferritin levels: 700 ng/ml (range 8-9033 ng/ml). Median interval between diagnosis and HSCT was 10 months (range 1-128 months). Time between diagnosis and HSCT was <6 months for 56 patients and ≥6 months for 166 patients. A total of 99 patients received standard conditioning (MAC) and 123 received a reduced intensity (RIC) regime. After conditioning 70 patients received stem cells from a sibling, 4 from a mismatched relative and 148 patients from an unrelated donor. The incidence of acute GvHD grade I-II and grade III-IV at 100 days was 1% (25-37%) and 14% (9-18%) respectively. The incidence of limited or extensive chronic GvHD at 36 months was 47% (40-54%) and 20% (15-26%) respectively.

Univariable analysis of relevant factors on primary end points

The median follow-up of the 222 registered patients after HSCT was 39 months with 95% confidence intervals (CI) ranging from 36 to 44 months. OS, RFS, NRM and RI at 36 months were 52% (95% CI: 45-59%), 44% (95% CI: 37%-51%), 25% (95% CI: 19%-32%) and 31% (95% CI: 24%-37%), respectively. Age had a significant impact on OS when analysed as continuous variable per decade (HR 1.4, 95%CI 1.1-1.8; p=0.002) also on RFS (HR 1.4, 1.1-1.8; p=0.002), and a borderline impact on NRM (HR 1.4, 1.0-1.8; p=0.05) and on relapse incidence (HR 1.3, 1.0-1.7; p=0.07). To evaluate the impact of WHO-classification on treatment outcome, patients were divided in 4 subgroups: patients with 5% marrow blasts or less at time of HSCT (RA, RARS, RCMD, RCMD-RS, 5q-), patients with 6-19 marrow blasts (RAEB1 and RAEB2), patients with ≥20 % marrow blasts (AML-MDS) and patients with CMML. The WHO-classification influenced significantly OS at 3 years (p = 0.002) (Table I,

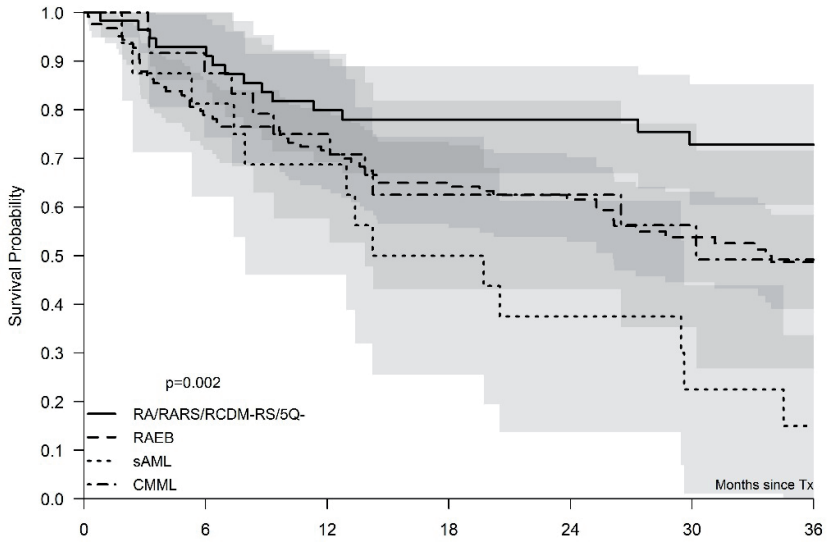
Table 1 demographic data and estimates of survival outcomes and cumulative incidences

	N	OS	NRM	RI	RFS
All patients	222	52% (45-59%)	25% (19-32%)	31% (24-37%)	44% (37-51%)
Median age (95% CI[@]) HR	59.3 (52.2-64.4)	1.4 (1.1-1.8) (0.002)	1.4 (1.0-1.8) (0.05)	1.3 (1.0-1.7) (0.07)	1.4 (1.1-1.8) (0.002)
Co-morbidities: overall p-value No Mild/moderate Severe	 126 (57%) 62 (28%) 34 (15%)	 0.02 57% (47-67%) 53% (40-66%) 33% (16-49%)	 0.1 21% (14-29) 26% (14-37) 40% (23-57)	 0.6 31% (22-39) 26% (15-38) 37% (20-54)	 0.03 48% (39-58%) 48% (35-61%) 23% (8-37%)
WHO at HSCT[x]: overall p-value RA/RARS/del5q/RCMD(RS) RAEB-1/RAEB-2 Secondary AML CMML	 57 (26%) 124 (56%) 16 (7%) 24 (11%)	 0.002 73% (60-85%) 49% (39-58%) 15% (0-34%) 49% (27-72%)	 0.5 21% (10-32) 28% (20-37) 25% (4-46) 17% (2-33)	 <0.001 7% (0 -14%) 34% (25-43) 69% (46-91) 42% (22-61)	 <0.001 72% (60-84%) 38% (28-47%) 6% (0-18%) 41% (21-61%)
Cytogenetic subgroups: overall p-value very good/good intermediate Poor/very poor^ Missing	 120 (58%) 40 (19%) 48 (23%) 14	 0.8 51 (41-61%) 60% (43-76%) 49% (33-65%)	 0.5 30% (21-38%) 20% (8-32%) 21% (8-33%)	 1 30% (21-38%) 34% (18-50%) 28% (15-42%)	 0.8 40% (31-50%) 46% (29-32%) 51% (36-66%)
Donor type: overall p-value Sibling Unrelated	 74 (33%) 148 (67%)	 0.7 52% (39-65%) 52% (43-60%)	 0.6 25% (14-35%) 26% (19-33%)	 0.9 28% (18-39%) 32% (24-40%)	 0.6 47% (35-60%) 42% (34-50%)
Sex match: overall p-value other combinations recipient-donor: m-f	 176 (79%) 46 (21%)	 0.2 55% (47-63%) 41% (25-57%)	 0.7 24% (18-31%) 28% (15-43%)	 0.2 30% (22-37%) 36% (22-50%)	 0.2 46% (38-54%) 36% (21-50%)
Conditioning Intensity Standard /Myelo-ablative Reduced Intensity	 99 (45%) 123 (55%)	0.1 55% (44-67%) 49% (39-58%)	0.6 25% (15-34%) 27% (19-35%)	0.2 25% (17-34%) 35% (26-44%)	0.3 50% (40-61%) 38% (29-48%)
RBCT* (median[@]): overall p-value 0-20 units >20 units Yes, but number not known Missing	 12 (5-29) 155 (70%) 49 (22%) 17 (8%) 1	 0.6 52% (44-61%) 51% (36-66%) 46% (16-75%)	 0.4 24% (17-31%) 28% (15-41%) 41% (12-70%)	 0.9 32% (24-40%) 29% (16-42%) 21% (0-44%)	 0.5 44% (36-52%) 43% (29-57%) 38% (8-67%)
Ferritin[#] ng/ml (median[@]): overall p-value ≤1000 ng/ml >1000 ng/ml Missing	(261-1554) 115 (58%) 81 (41%) 26	 0.09 57% (48-67%) 46% (35-58%)	 0.3 22% (14-30%) 28% (18-39%)	 0.3 27% (19-35%) 36% (25-47%)	 0.1 51% (42-61%) 36% (25-47%)
C-reactive protein (median[@]): overall p-value Not elevated Elevated Missing	 (2.2-10.1) 146 (75%) 49 (25%) 27	 0.02 55% (47-64%) 42% (27-56%)	 0.1 22% (15-29%) 33% (19-46%)	 0.6 33% (25-41%) 28% (15-42%)	 0.4 45% (36-54%) 39% (24-53%)
Chelation (yes): overall p-value Yes No Missing	 31 (31%) 70 (69%) 152	 0.6 44% (21-67%) 45% (33-57%)	 0.9 33% (13-53%) 29% (18-40%)	 0.9 34% (15-53%) 33% (22-44%)	 0.9 33% (13-54%) 38% (26-50%)

Estimates of overall survival (OS), non-relapse mortality (NRM), relapse incidence (RI) and relapse-free survival (RFS) at 3 years after HSCT, expressed as percentage with 95% confidence intervals. Differences between outcomes until 3 years after HSCT for subgroups were evaluated by the log-rank test (OS and RFS) and the Gray test (RI and NRM);

@: median (1st-3rd Qu); [x]missing 1 patient; *: number of red blood cell transfusions (RBCT) prior to HSCT; # just prior to conditioning HSCT; ^: only 9 patients had very poor cytogenetic characteristics. Therefore, poor and very poor risk groups are lumped together.

Figure 1. Cytogenetics according to IPSS-R categories, had no significant impact on treatment outcome. However, the group with very poor-risk cytogenetics could not be analysed separately due to its small size (n=9) (details provided in Table I). Only 11 patients were transplanted with bone marrow as stem cell source. Therefore, the impact of stem cell source has not been analysed separately. Transfusion burden (≤20 RBCT versus >20 RBCT) did not have a significant impact on any of the outcome parameters. Ferritin levels (>1000 ng/ml) had a non-significant negative impact on NRM and OS after HSCT, but elevated CRP levels (> 10 mg/l) influenced OS significantly (p=0.02).



N at risk							
RA/RARS/RCDM-RS/5Q-	57	50	43	40	35	28	23
RAEB	124	97	88	77	65	44	37
sAML	16	13	11	8	6	3	2
CMML	24	21	18	15	14	8	6

Figure 1 Overall survival according to WHO Categories. Kaplan-Meier plot for OS in the whole cohort, grouped by WHO categories. The corresponding 95% confidence intervals are indicated by the gray regions. The Log-rank p-value is indicated.

Correlations of potentially confounding factors for the multivariable analyses
Patients with more than 5% bone marrow blasts received a low number of red blood cell units (0-20 RBCT) before HSCT more frequently than patients with less than 5% bone marrow blasts (p = 0.01), mainly explained by a shorter duration of the interval between diagnosis and HSCT in patients with more advanced MDS according to WHO-classification (data not shown). The number of RBCT before HSCT was not associated with the cytogenetic risk category according to IPSS-R (p = 0.7) nor with interval between diagnosis and actual HSCT of the whole cohort, including patients with less than 5% marrow blasts (<6 vs >6 months) (p = 0.2). As expected, ferritin levels (>1000 ng/ml versus <1000 ng/ml) were influenced by number of administered RBCT prior to HSCT (p<0.001). Ferritin levels, measured as units of 1,000 mg/L were associated with elevated CRP levels (p=0.03) in this study. Co-morbidities and intensity conditioning (MAC versus RIC) were significantly associated (p<0.001).

Multivariable Cox models
The basic model was restricted to the traditional variables indicating a potential prognostic impact, as known from the literature and as indicated by the univariable analyses (Table II). Therefore, only WHO-classification, age at HSCT, donor type, sex-match, and intensity of conditioning regimen were included in the model. We added one by one the other variables of specific interest in the current study: RBC transfusions, CRP levels, ferritin levels (continuous in units of 1000 ng/ml) and comorbidities. This approach was necessary due to the highly correlated nature of these variables. The transfusion burden prior to HSCT influenced RFS significantly (HR 1.7; p = 0.02). The impact of pre-HSCT ferritin levels was minor but significant on OS (HR 1.2; p = 0.05) and RI (HR 1.3; p = 0.04). Presence of severe comorbidities influenced OS after HSCT (HR 1.8; p 0.04). Elevated CRP-levels (>10 mg/L) had a borderline negative impact on survival (HR 1.6; p=0.06).

Impact of iron chelation before HSCT on outcome
Thirty-one (14%) patients received iron chelation prior to HSCT and ferritin levels at HSCT were available in 28 patients of them. The median duration of chelation prior to HSCT was 4 months (range: 0 to 40 months). The median ferritin levels at HSCT was 1598 ng/ml, 9 chelated patients had ferritin levels < 1000 ng/ml at time of HSCT. Sixteen chelated patients had received >20 units of RBCT prior to HSCT. The outcome of the patients chelated prior to transplant conditioning was compared with a non-chelated control group of 70 patients who had ferritin levels >1000 ng/ml at HSCT or who had received >20 units of RBCT prior to HCT conditioning. The overall 3-year survival in the chelated group was 44% (21-67%) similar to the survival in control group 45% (33-57%, p = 0.6; Figure 2A). The RFS, RI and NRM were also similar in both groups: 33% (13-54%) in the chelated patients versus 38% (26-50%) in the non-chelated patients, 34% (15-53%) versus 33% (22-44%), and 32% (13-53%) versus 29% (18-40%), respectively (Figure 2B).

Table 2 Multivariate Cox models for assessment of impact of relevant factors for all major outcomes.

	OS HR (95% CI) (p)	NRM HR (95% CI) (p)	RI HR (95% CI) (p)	RFS HR (95% CI) (p)
Basic model				
Age (decades)	1.4 (1.1-1.7) (0.01)	1.4 (1.0 – 2.0) (0.04)	1.1 (0.8 – 1.4) (0.73)	1.2 (1.0 – 1.5) (0.09)
WHO classification <5% marrow blasts@ RAEB/CMML Transformed AML	 1 1.8 (1.0-3.2) (0.05) 3.0 (1.4-6.6) (<0.001)	 1 1.3 (0.7-2.6) (0.4) 1.4 (0.5-4.6) (0.5)	 1 5.5 (2.0-15.3) (<0.001) 14.3 (4.4-47) (<0.001)	 1 2.4 (1.4-4.2) (<0.001) 4.6 (2.2-9.6) (<0.001)
Patient – donor sex Other combinations Female donor into male recipient	 1 1.2 (0.8-2.0) (0.4)	 1 1.3 (0.7-2.5) (0.5)	 1 1.2 (0.7-2.2) (0.5)	 1 1.3 (0.8-1.9) (0.3)
Conditioning Myeloablative Reduced Intensity	 1 1.2 (0.8-1.9) (0.3)	 1 1.1 (0.6-2.0) (0.7)	 1 1.4 (0.8-2.3) (0.2)	 1 1.3 (0.9-1.8) (0.3)
Added to basic model				
Transfusions RBCT ≤ 20 units RBCT >20 units	 1 1.5 (0.9-2.5) (0.09)	 1 1.7 (0.9-3.4) (0.1)	 1 1.8 (0.95-3.3) (0.07)	 1 1.7 (1.1-2.7) (0.02)
C-reactive protein CRP ≤ 10 CRP > 10	 1 1.6 (1.0-2.5) (0.06)	 1 1.5 (0.8-2.8) (0.2)	 1 0.8 (0.4-1.5) (0.5)	 1 1.1 (0.7-1.7) (0.8)
Ferritin (units of 1,000 ng/ml)	1.2 (1.0-1.4) (0.05)	1.1 (0.8, 1.4) (0.6)	1.3 (1.01-1.6) (0.04)	1.2 (0.98-1.4) (0.08)
Comorbidities No Mild/Moderate Severe	 1 0.9 (0.6-1.6) (0.8) 1.8 (1.03-3.0) (0.04)	 1 1.1 (0.6-2.2) (0.8) 2.0 (0.97-4.0) (0.06)	 1 0.7 (0.4-1.3) (0.3) 1.4 (0.7-2.7) (0.4)	 1 0.9 (0.5-1.3) (0.5) 1.6 (0.98-2.6) (0.06)

@: including RA/RARS/del5q/RCMD(RS); Patients with missing data for a variable were excluded from the models where this variable was analysed.

Impact of phlebotomies or iron chelation after HSCT on outcome

Twenty patients have been treated with phlebotomies after HSCT. Most patients (80%) started phlebotomies during the first year after HSCT. The median ferritin levels before starting phlebotomies was: 3134 ng/ml (range: 937 – 9985 ng/ml). The number of phlebotomies was available in 16 patients: 9 patients underwent <10 phlebotomies and 7 patients 10 or more phlebotomies. The ferritin levels decreased to levels <1000ng/ml in 10 out of 19 patients at time of completion of phlebotomies or at 24 months after HSCT and decreased more than 50% in five additional patients. In the remaining 5 patients the effect was not evaluable mainly due to insufficient follow-up after starting phlebotomies.

Sixteen patients have received iron chelation (deferasirox only) after HSCT. Nine patients started chelators during the first 6 months after HSCT and 13 patients during the first year after HSCT. The median interval between HSCT and starting chelation was 5 months (range: 1-18). The median ferritin level before starting chelation was: 3122 ng/ml (range: 69 – 9040 ng/ml). The median duration of iron chelation was 4 months (range: 0.5 to 40 months). The ferritin levels decreased to levels <1000 ng/ml in 3 out of 16 patients during iron chelation and decreased more than 50% in four additional patients. In the remaining patients the effect was not evaluable due to missing ferritin levels (5 patients) or too short duration of chelation (7 patients). The median duration of iron chelation in the 5 responding patients was 4 months (range: 1.5 to 7 months).

We combined the data of both interventions, because the number of patients who were treated with either chelation or phlebotomies was relatively small, and both interventions are aiming at reducing the iron overload. Fourteen patients started treatment with either phlebotomies or chelation during the first 6 months after HSCT, and 27 patients started these interventions within the first 12 months after HSCT. The outcome of the patients treated with iron reductive interventions was compared with the untreated control groups with ferritin levels >1,000 ng/ml alive and relapse free at the landmarks of 6, 12 and 24 months respectively (Table III). Only patients who received iron reductive therapy within 6 months after HSCT had a significantly improved 3 year RFS compared to the non-treated patient population (90% (71-100%) vs 56% (46-67%); p=0.04 Figure 3, table III). The survival at this landmark analysis was moderately reduced compared to the control group with an 3-year OS of 90% (71-100%) versus 65% (54-75%) in the control group (Table III). The outcomes at later landmark analyses did not indicate relevant differences with the exception of a moderate difference of the NRM at both landmarks (Table III).

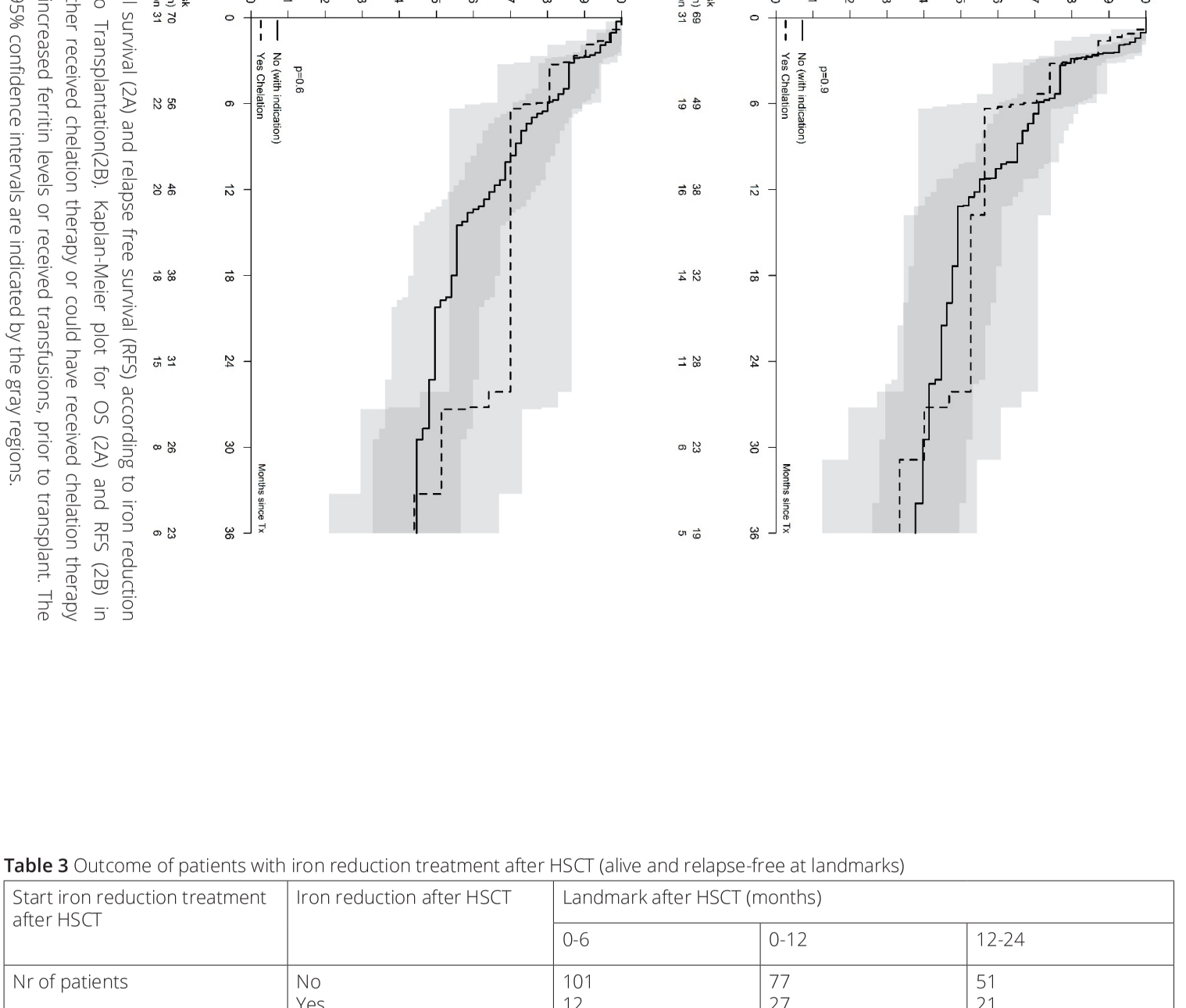


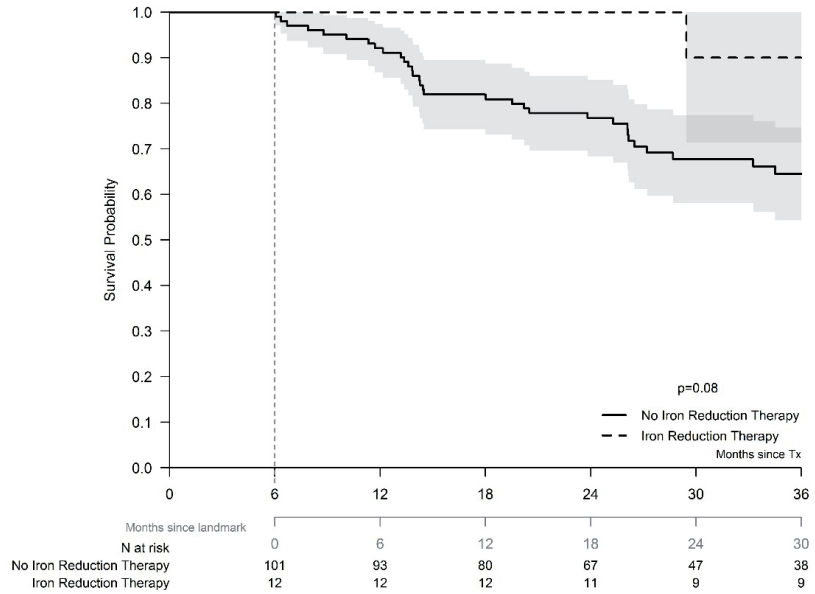
Figure 2 Overall survival (2A) and relapse free survival (RFS) according to iron reduction therapy prior to Transplantation(2B). Kaplan-Meier plot for OS (2A) and RFS (2B) in patients who either received chelation therapy or could have received chelation therapy based on their increased ferritin levels or received transfusions, prior to transplant. The corresponding 95% confidence intervals are indicated by the gray regions.

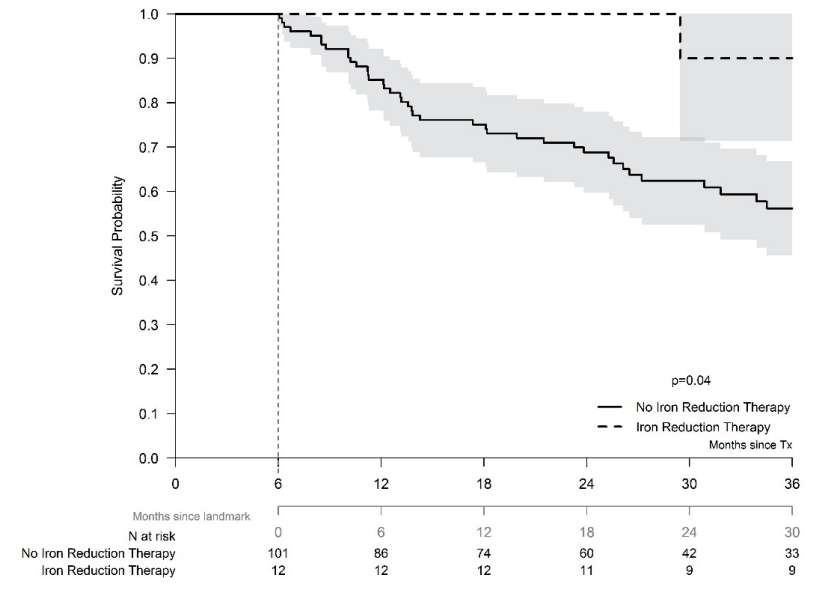
Table 3 Outcome of patients with iron reduction treatment after HSCT (alive and relapse-free at landmarks)

Start iron reduction treatment after HSCT	Iron reduction after HSCT	Landmark after HSCT (months)					
		0-6		0-12		12-24	
Nr of patients	No Yes	101 12		77 27		51 21	
OS#	No Yes	65% (54-75%) 90% (71-100%)	0.08	75% (65-86%) 81% (61-100%)	0.3	85% (79-98%) 88% (73-100%)	0.9
RFS#	No Yes	56 (46-67%) 90% (71-100%)	0.04	67% (55-79%) 57% (58-98%)	0.3	81% (69-93%) 88% (74-100%)	0.5
Relapse Incidence#	No Yes	26 %(17-35%) 10% (0-29%)	0.19	18% (8-27%) 22% (2-42%)	0.8	7% (0-16%) 12% (0-26%)	0.6
NRM#	No Yes	18% (10-26%) 0% (0-0%)	0.13	15% (6-24%) 0% (0-0%)	0.08	12% (2-21%) 0.14 0% (0-0%)	

* Control group: patients with ferritin levels above 1000 ng/ml at start comparison
#: estimates of outcomes at 3 years after HSCT (95% confidence intervals) with p-values of log rank test/Gray test of non-treated vs treated groups. The outcomes are measured from the three landmark points (6, 12 and 24 months after HSCT)
Separate table describing relevant characteristics of both groups at 6 and 12 months (see, supplementary files): mean age, WHO classification (3 groups), RBCT < vs >20 units, sever co-morbidities, severe cGVHD

Figure 3 Overall survival and relapse-free survival of patients alive and relapse-free at 6 months after transplantation, stratified in 2 groups according to iron reduction therapy given during the first 6 months after transplantation or not. Landmarked Kaplan-Meier plot for OS and RFS in patients who either received iron reduction therapy or could have received iron reduction therapy based on their increased ferritin levels or received transfusions. Patients were selected based on ferritin levels >1000 ng/ml**.** The landmark time-point is indicated by the vertical dashed line. Only patients alive and relapse-free at 6 months are included. The corresponding 95% confidence intervals are indicated by the gray regions.





Discussion

Allogeneic haematopoietic stem cell transplantation (HSCT) is considered the most potent curative option in myelodysplastic syndromes (MDS). Many retrospective analyses evaluated relevant prognostic parameters, to enable adequate patient selection for treatment[16,17] and reviewed in.[18] This large prospective observational study collected detailed information on several relevant prognostic factors, including specific patient characteristics, disease modalities, transfusion data and iron parameters in patients with MDS undergoing HSCT. Age, BM blast percentage and comorbidity at time of HSCT had a significant influence on outcome after HSCT. The data of this study showed that intensity of conditioning regimen had no impact on overall survival, confirming several other retrospective studies.[5,6,19,20] The primary objective of this study was to study the impact of iron overload and iron toxicity on outcome after HSCT. As expected[21], the data showed a significant association between transfusion burden and ferritin levels (p<0.001). A high RBCT burden, higher than 20 units prior to transplantation, was significantly associated with a decreased relapse-free survival (HR 1.7; P=0.02). Although it is difficult to separate the impact of transfusion load as an independent prognostic marker from progression of bone marrow failure and increased tumour burden, several studies showed a significant impact of transfusion burden on HSCT outcome[17,22,23], including our own retrospective study.[24] In this study the impact of high transfusion burden on overall survival was less explicit (HR 1.5; P=0.09), in contrast to the significantly reduced RFS, which can be explained by the smouldering nature of less advanced MDS patients in this cohort (only 16 patients had AML-MDS at time of transplantation). This may result in a prolonged survival after relapse.

Ferritin is an acute-phase protein, elevated in case of infection, inflammation or high tumour burden.[25] To correct for this confounding factor we analysed CRP levels, another acute phase protein, which was significantly correlated with ferritin levels. Both ferritin levels and elevated CRP levels had an impact on overall survival, but only ferritin levels had an significant impact on relapse incidence. On the other hand, the impact of elevated CRP levels on NRM was more pronounced compared to the impact of elevated ferritin levels, confirming data from a large HSCT study in AML and MDS.[26] Ferritin levels are sometimes used as a marker of transfusion burden,[27] but in the multivariable analysis of that study only co-morbidities and percentage of marrow blasts at time of HSCT predicted OS.[27] Therefore, it has been postulated that transferrin saturation, NTBI and LPI are better parameters to monitor and to study iron toxicity during conditioning for HSCT and the first two weeks after HSCT.[28] When transferrin saturation exceeds 85%, species of non-transferrin-bound iron (NTBI), and its redox active component labile plasma iron (LPI), become detectable in the plasma.[29,30] Transferrin becomes

saturated immediately during transplant conditioning and remains elevated until engraftment.[30] These redox active components lead in various steps to associated tissue damage, mostly cardiac, endocrine and liver tissue and may also affect clonal evolution in MDS.[31] Reduction of iron stores, may alleviate long-term effects of iron toxicity in transfusion dependent patients with MDS.[32] Consensus statements advice to administer iron chelation therapy in those patients with an expected long-term survival.[33,34] In high-risk MDS, patients might not live long enough to endure the toxic effects of transfusional therapy. Research of the impact of iron chelation in a transplant setting is ongoing.[35]

In the current study we were able to investigate the impact of iron reduction therapy on outcome after HSCT. Iron overload in the transplant setting may be reduced both by iron chelation therapy[36,37] and by phlebotomies.[38] Administration of iron chelation therapy prior to HSCT had no influence on treatment outcome in this study. However, the ferritin levels of the chelated patients at HSCT were still elevated with a median level of 1772 ng/ml, and only 8 out the 28 chelated patients with available ferritin levels had ferritin levels <1000 ng/ml at HSCT. This indicates that iron chelation was insufficient in the majority of cases, probably reflecting the short period of iron chelation prior to HSCT. Patients with MDS may also receive iron reduction interventions to reduce iron overload after HSCT.[39] We were able to evaluate the influence of iron reduction therapy after HSCT in a subgroup of 35 patients, consisting of iron chelation in 16 patients and/or phlebotomies in 20 patients (one patient received bot phlebotomies and iron chelation). Iron reduction by phlebotomies started within one year after HSCT in 80% and by iron chelation in 81% of the patients receiving the respective therapies. The efficacy of reducing ferritin levels by phlebotomies was excellent with more than 50% reduction in 74% of the patients and 28% after chelation, probably reflecting the short period of chelation (median 4 months). Most events (relapse or death by any cause) occurred within the 6 months after transplantation in our study. Therefore, iron reduction therapy is expected to have more impact on outcome, if initiated within 6 months after HSCT, as shown in our study. The patient population who received iron chelation therapy or phlebotomies within 6 months after HSCT had a significantly improved RFS compared to non-chelated patient population with ferritin levels >1000 ng/ml or having received transfusions before HSCT (P = 0.04), while the OS at this landmark was moderately (P=0.08) improved. Selection of the patients for either of the interventions cannot be ruled out, but the data in the supplementary table do not show remarkable differences in the both groups.

In conclusion, analysis of this non-interventional prospective data from MDS patients, not pre-treated with intensive chemotherapy before undergoing allogeneic HSCT, demonstrated that transfusion burden prior to HSCT influenced progression free survival, without a significant effect on survival. Outcome might be predicted by serum ferritin levels, irrespective to CRP levels. Administration of iron reduction prior to HSCT had no impact on primary outcome, but iron reduction therapy after HSCT increased relapse-free survival, if initiated within 6 to 12 months after HSCT.

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E.C. coordinated the study, analysed the data and wrote the paper. T.d.W. analysed the data, designed the research, and co-authored the paper. L.d.W., D.E. and D.Z performed the statistical analysis, and co-authored the paper. E.K and A.v.B. collected the data. J.F., D.B., G.S., A.N.., G.K., L.V., T.G., J.S., Y.B., G.S., P.L., and A.A., admitted a substantial amount of patients and critically reviewed the paper. M.R. and N.K. chaired the CMWP meetings during which the progress of the study and this manuscript was discussed, they also reviewed the paper.

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CHAPTER 10

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

General discussion and future perspectives

Myelodysplastic syndromes is a heterogeneous group of clonal hematopoietic bone marrow disorders, characterized by cytopenias and increased risk of leukemic transformation. The diagnosis is established on the presence of specific cytomorphological (CM) findings, the presence of MDS-associated cytogenetic abnormalities or presence of a mutation in the splicing chromosome *SF3B1* (in the context of presence of ring sideroblasts).[1–3] In patients with cytopenia(s), distinction between clonal and non-clonal diseases can be challenging due to many reasons. On one hand, MDS cases might present with minimal dysplastic features, no increased percentage of blasts and normal cytogenetic analysis. On the other hand, dysplasia is not restricted to MDS, it is also seen in reactive conditions. Furthermore, cytogenetic abnormalities are absent in around 50% of the MDS cases and rarely MDS-specific.[4] The diagnostic guidelines for MDS established by the European LeukemiaNet (ELN) recommend the addition of FC, and suggest additional molecular techniques to support the diagnosis of MDS.[5] As described in the first two chapters FC is ready for clinical implementation in the diagnostic work-up in MDS, especially in excluding clonal disorders in patients with minimal dysplastic features and normal cytogenetics. The specificity (true negative) of MDS-FC analysis calculated after one year follow-up was 95%. This means that MDS-FC was very specific in excluding development of MDS within one year. The aim of the first part of this thesis was to evaluate and improve currently applied MDS-FC algorithms and investigate the efficacy of novel diagnostic tools in the diagnostic work-up in MDS.

This thesis provides recommendations for improvement of current applied MDS-FC panels. The first step was to review currently used MDS-FC approaches. Overall, the most commonly used diagnostic MDS-FC models have a sensitivity of 71% and specificity of 93%.[6,7] Due to wide availability of CD-markers that describe the granulocytic differentiation and maturation, this cell compartment forms the cornerstone of FC algorithms.[8,9] Our study focused on the differences between MDS and pathological controls, all patients with cytopenias. The most concise panel for proper distinction between these patient groups included: CD34, CD117, CD11b, CD13, CD16 and CD56. Most markers are already present in the proposal for AML/MDS analysis by the EuroFlow Consortium. In combination with CD56, this might provide a highly specific MDS-FC panel. The sensitivity of these parameters can be calculated after addition of the other cell compartments, as MDS is not diagnosed solely based on abnormal neutrophils. The lower sensitivity (around 71%) of most commonly used diagnostic MDS-FC models is partially caused by the absence of the erythroid cell compartment in these models. Therefore, we developed and validated the erythroid lineage evaluation and added it to applied

MDS-FC algorithms. Only four parameters based on three markers (CD117, CD71, CD36) were sufficient to achieve an increased diagnostic sensitivity to 80%, without effecting the specificity (95%). To further increase the sensitivity from MDS-FC the megakaryocytic lineage should be added. Evaluation of the megakaryocytic lineage still faces technical challenges. However, the evaluation of CD34, CD36, CD42a an CD61 are very promising. [10] Note that this means that addition of only 2 markers to the panel suggested above is sufficient. The megakaryocytic evaluation is currently under development within the ELNet MDS Flow Cytometry

To keep, and improve the specificity, the same analysis as preformed for the granulocytes should be performed for the monocytes. There are multiple markers, that describe the monocytic cell population. The challenge here is to identify markers that distinct MDS from other clonal diseases such as chronic myelomonocytic leukemia and myeloproliferative disorders. Others suggest panels including CD14, CD16, HLA-DR, CD64, CD56.[8,11]

Overall, evaluation of endless lists of CD-markers should be avoided. Shorter panels that evaluate the erythroid, myeloid progenitors, neutrophils, monocytes, and megakaryocytes, will lead to an overall higher specificity. FC can underline the diagnosis in indifferent cases, or even predict development of MDS in the near future. The role of other cell subsets such as dendritic cells (although very promising), and lymphocytes (except progenitor B cells) still need further investigation before introducing them in current standardized diagnostic panels, as their role in the identification of MDS is unclear. Aim should be to develop a standardize single tube multicolour FC analysis, which can be performed widely, and does not require high levels of expertise. Here new software can help to simplify the procedures. Panels need to be concise, easier to apply, less time consuming and cheaper.

Flow cytometry is described as an additional diagnostic tool to complement CM and cytogenetic analysis in suspected MDS. Note that, FC and CM evaluate different cell properties/aspects/features and therefore not necessarily correlate with one another. A big challenge in this thesis, and also in other studies regarding FC in MDS, is that FC results are always correlated to the gold standard. And here, the gold standard is CM features, in some cases supplemented by genetic abnormalities. Note that the goal of the current research is not to replace CM by other techniques but to complement or improve the diagnostic work-up. Because, as we demonstrate in Chapter 6, the current diagnostic subgroups described by the WHO classifications are very heterogeneous in many aspects (features described by the different tools). Therefore, we like to suggest that for future research of novel diagnostic tools such as FC, SNP-arrays, and next-generation sequencing, results should be correlated to other parameters such as clinical

features (i.e. presence of cytopenias, auto-immune phenomena, etc.) or prognosis (overall survival, time until leukemic evolution, therapy response, etc.).
Other pitfall in MDS analysis is that MDS is a clonal disease, and during the course of the disease the clonal cell population modifies due to disease, patient or treatment influences. Here studies that compare early onset MDS patients (based on high specific MDS-FC models that predict clonal development) to high risk highly aberrant MDS patients are needed. Also long-term follow-up of patients is mandatory to gain more insights in disease development. Identification of type, function, and moment of occurrence of a certain mutation with or without abnormal phenotype will provide better models that can predict prognosis or even treatment response.
By exploring the data in chapter 6, we found correlations between specific FC aberrancies and mutational status irrespective to the WHO diagnosis. We found that mutations in epigenetic regulators and transcription factors lead to aberrant myeloid progenitors, granulocytes or monocytes according to FC. What is the clinical impact of these findings? Research on pathophysiological mechanisms behind these correlations is mandatory as it will provide new targets for therapy. As MDS is a clonal disease that changes over time, a single drug approach seems not sufficient. Techniques that can monitor clonal evaluation might guide treatment discussions: if the clone starts to appear again, or starts changing a different therapeutic strategy is mandatory. Here FC has already proved its applicability in acute myeloid leukemia, but also other techniques such as next generation sequencing or mass cytometry are thinkable.[12] However, these are complicated often expensive tools that are not yet ready for general application. SNP-array might be a good alternative as it becomes more widely available by commercial platforms.

Treatment options in MDS include growth factors (erythropoietin or G-CSF), hypomethylating agents (decitabine or azacitidine), or immune-modulating drugs (lenalidomide). The only potent curative option in MDS is an allogeneic hematopoietic stem cell transplantation (allo-SCT), upfront or after cytoreduction[13]. Although reduced intensity have decreased non-relapse mortality, overall survival remains around 30%. To improve allo-SCT outcome, more research needs to be performed in optimizing therapeutic strategies. As part of supportive care, most MDS patients receive multiple red blood cell transfusions (RBCT) during the course of their disease. Multiple transfusion can cause secondary hemochromatosis (reviewed in Chapter 7), a deleterious effect of iron accumulation in heavily transfused MDS patients. In the following chapters we evaluate the influence of RBCT on outcome in allo-SCT.

In chapter 8 we moved scenery to prognosis in high risk myelodysplastic syndromes (MDS) and treatment with allo-SCT. Due to high treatment-related morbidity and mortality and high relapse risk the current long-term overall survival rate is around 30%.[14] Over the years reduced intensity regimens were introduced for the elderly and more frail patients to reduce non-relapse mortality. However, treatment selection and treatment timing remains challenging. Many retrospective analysis evaluated relevant prognostic parameters. In these studies MDS patients are often pre-treated, which leads to very heterogeneous patient cohorts in respect to patient and disease characteristics. To diminish these confounding factors the Chronic Malignancies Working Party of the EBMT analysed MDS patients treated by upfront allo-SCT, who were not extensively pre-treated. This part of the thesis aimed to identify prognostic parameters that aid treatment outcome prediction and assist patients selection for treatment with allo-SCT. The focus here, the influence of RBCT and its associated iron overload and iron toxicity. The retrospective analysis in chapter 9 formed the rational for the prospective non-interventional study in chapter 10. The prospective analysis concluded that transfusion burden influenced progression free survival, without a significant effect on survival. Treatment outcomes after 36 months in this not intensively pre-treated patient cohort were 52% overall survival (95% CI: 45%-59%), 44% relapse-free survival (95% CI: 37%-51%), 26% non-relapse mortality (95% CI: 19%-32%) and 31% relapse incidence (95% CI: 24%-37%). Expected parameters such as age, blasts percentage and comorbidity had a significant influence on outcome after ALLO-SCT.[13] As also recently described in another large patient cohort, regimen intensity had no impact on overall survival in this study.[15] Outcome might be predicted by serum ferritin levels, irrespective to CRP levels. Administration of iron reduction prior therapy prior to allo-SCT had no impact on primary outcome, but iron reduction therapy during the first year after allo-SCT increased overall survival. This thesis suggested the reduction of transfusion related iron overload in the allo-SCT setting. The easiest way to achieve this, is to reduce transfusions amount by shortening interval between diagnosis and curative therapy. The prediction of time of diagnosis until to leukemic evolution is difficult by current prognostic models. Development of better prognostic models, will lead to better treatment decisions (type of therapy and timing of starting therapy).

In summary, the thesis illustrates the efficacy of conventional and novel diagnostic tools in MDS. We provided suggestions for improvement of currently implied MDS-FC algorithms with the suggestion of a minimal panel and by reducing number of granulocytic markers and by the addition of the erythroid lineage. We illustrated the disease heterogeneity within MDS with the application of different diagnostic tools. And provide suggestions to improve disease analysis in the

future. The second part the thesis illustrated the deleterious effect of secondary hemochromatosis on long term survival in MDS patients treated with best supportive care and especially the negative impact in high risk MDS patients that undergo allogeneic ALLO-SCT. Here, a positive impact of iron reduction therapy was suggested.

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CHAPTER 11

NEDERLANDSE SAMENVATTING VOOR NIET MEDISCHE INGEWIJDEN

Nederlandse samenvatting voor niet medische ingewijden

Het myelodysplastiche syndroom (MDS) is een groep beenmergziekten met een variabel beloop, van een zeer langzame verslechtering tot zeer snelle progressie naar acute myeloïde leukemie. De verschillende typen MDS worden gekarakteriseerd door een gestoorde aanmaak en een gestoorde uitrijping van bloedcellen. Deze aanmaak en uitrijping van voorlopercellen tot bloedcellen vindt plaats in het beenmerg. MDS patiënten kampen dan ook met de gevolgen van een tekort aan bloedcellen: vermoeidheid (veroorzaakt door tekort aan rode bloedcellen), bloedingen (veroorzaakt door een tekort aan bloedplaatjes) of steeds terugkerende infecties (door een tekort aan witte bloedcellen). De geschatte incidentie van MDS is 5,4 nieuwe gevallen per 100.000 personen in de Nederlandse populatie. Deze incidentie neemt toe met de leeftijd, tot 84,4 per 100.0000 rond het 80ste levensjaar.

Diagnose

De diagnose wordt gesteld op basis van onderzoek van het beenmerg en het perifere bloed. Na kleuring van de cellen, wordt met een microscoop de cel-uitrijping beoordeeld. De diagnose MDS kan worden gesteld wanneer er sprake is van meer dan 10% dysplastische (afwijkend uitrijpende) cellen in een of meerdere cellijnen, of wanneer er sprake is van ring sideroblasten (cellen met een afwijkende ijzerkleuring) in meer dan 15% van de rode bloedcel-voorloper cellen. Daarbinnen kan er sprake zijn van: MDS met uniliniaire dysplasie (afwijking in één cellijn), of MDS met multiliniaire dysplasie (afwijking in twee of meer cellijnen), al dan niet met ring sideroblasten. Aanvullend wordt chromosoomonderzoek verricht (cytogenetica). Bij ongeveer 50% van de MDS patiënten worden namelijk genetische afwijkingen gevonden die helpen bij het stellen van de diagnose, alsook iets zeggen over de prognose (ziektebeloop). In de laatste versie van de WHO-classificatie (World Health Organization: richtlijnen voor diagnostische classificatie) voor MDS wordt ook geadviseerd om specifiek te kijken naar een mutatie in het *SF3B1*-gen.
De diagnose MDS is niet altijd eenduidig te stellen. Zeker niet bij patiënten met cytopenie (cellen tekort) waar maar weinig dysplasie in het beenmerg gevonden wordt en er geen chromosomale afwijkingen zijn. In deze gevallen kan toevoeging van een andere laboratoriumtechniek, de zogenaamde flowcytometrie, uitkomst bieden. Zo laten we in hoofdstuk 2 van dit proefschrift zien dat flowcytometrie in deze gevallen MDS definitief kan uitsluiten.

Flow cytometrie

Flowcytometrie is een techniek die kan worden gebruikt voor het bestuderen van bepaalde celeigenschappen; met als doel cellen op basis van hun celkenmerken te sorteren. In MDS wordt flowcytometrie gebruikt om te kijken naar verdeling van de verschillende soorten bloedcellen, celgrootte (voorwaartse lichtverstrooiing) en aanwezigheid van korrels in de cel (granulatie; zijwaartse lichtverstrooiing). Bovendien kan de uitrijping van de witte en rode cellijnen worden bestudeerd. In hoofdstuk 3 van dit proefschrift evalueren we de analyse van de granulocyten (een vorm van witte bloedcellen), die zoals gezegd een belangrijke rol hebben binnen MDS diagnostiek. Dit komt doordat deze populatie relatief groot is en er veel flowcytometrie-merkers (CD-nummers) beschikbaar zijn om de cellen te onderscheiden. De meeste markers worden aangeduid met een CD-nummer, bijv. CD45 en CD34. CD45 herkent alle witte bloedcellen en CD34 alleen de hele vroege bloedvormende cellen. Een combinatie van flowcytometrie-merkers die getest wordt heet een flowctometrie-panel. Door een selectie te maken van de meest informatieve merkers, kunnen flowcytometrie-panels korter worden, dit betekent minder werk en goedkoper. Dit zonder dat het ten koste gaat van de specificiteit en sensitiviteit van flowcytometrie. De algemene specificiteit en sensitiviteit van MDS-flowcytometrie is ongeveer 93% en 71%, d.w.z. in 93% gevallen zegt FC terecht dat de diagnose anders is dan MDS, in 71% van de gevallen klopt de diagnose MDS met de microscoopbeoordeling.

De meeste MDS patiënten zullen kampen met de gevolgen van bloedarmoede, een tekort aan rode bloedcellen. De huidige MDS-flowcytometrie modellen omvatten echter niet de analyse van voorloper rode bloedcellen. In dit proefschrift voegen we de analyse van de rode cellijn toe aan de gebruikte modellen, waarop de sensitiviteit van MDS-flowcytometrie toeneemt tot 80%. De specificiteit blijft hiermee gelijk aan die van de bestaande MDS-flowcytometrie modellen (~95%) (Hoofdstuk 4-5).

Zoals gezegd is MDS een gevarieerd ziektebeeld, waarbij het moeilijk blijft te voorspellen of en wanneer een patiënt een leukemie zal ontwikkelen. Om meer inzicht te krijgen in dit ziektebeeld en de ontwikkeling, analyseren we in hoofdstuk 6 een groot patiëntencohort met laag risico MDS waarbij we verschillende diagnostische technieken gebruiken. Naast de morfologie, cytogenetica en flowcytometrie, gebruiken we hier ook SNP-array (een manier om naar DNA foutjes te kijken) en next generation sequencing (een techniek om grote stukken DNA te analyseren). Hier tonen we een divers landschap met velerlei aberranties (o.a. op basis van flowcytometrie) en mutaties, die niet correleren met de huidige diagnostische indeling van de WHO. Waarbij we dus kunnen stellen dat de door ons onderzochte technieken aanvullende waarde kunnen hebben in de diagnostiek van MDS. Mogelijk kan ons onderzoek ook aanvullend zijn aan de WHO-classificatie voor het voorspellen van ziektebeloop en voor respons op therapie (dit werd niet verder onderzocht).

Prognose
Om een keuze te maken voor een type behandeling moet er eerst een inschatting gemaakt worden van het ziektebeloop. Patiënten waarvan namelijk verwacht wordt dat zij snel progressie naar acute myeloïde leukemie zullen vertonen, zullen eerder en intensiever moeten worden behandeld. Een model dat hiervoor is ontwikkeld, is het International Prognostic Scoring System (IPSS), die in 2012 werd gereviseerd (IPSS-R). De IPSS-R bestaat uit: het percentage aan jonge voorloper cellen (blasten) in het beenmerg, chromosomale afwijkingen, het aantal en de diepte van de celtekorten. Afhankelijk van de prognostische waarden, worden punten toegekend. Deze score verdeelt patiënten in 5 subgroepen: 'heel laag', 'laag', 'gemiddeld', 'hoog' en 'heel hoog'. Deze subgroepen correleren met een algemene overleving van 8,8 jaar tot 0,8 maanden en de tijd tot leukemische ontaarding van nooit tot 0,7 maanden.

Eerdere studies hebben laten zien dat flowcytometrie in staat is om prognostische subgroepen aan te tonen binnen de vijf IPSS-R subgroepen. Betere prognostificatie kan leiden tot betere therapiekeuze. Een patiënt waarvan verwacht wordt dat hij niet gaat transformeren naar een acute myeloide leukemie kan zo therapie bespaard blijven. Bovendien kan een patiënt met een heel hoog risico op leukemische transformatie veel sneller in aanmerking komen voor een allogene stamceltransplantatie. Het diagnostische model dat in hoofdstuk 6 beschreven wordt, zou kunnen dienen als basis voor een prognostisch model.

Behandeling
De enige genezende behandeling voor MDS patiënten is het ondergaan van een allogene stamceltransplantatie, waarbij de patiënt beenmergcellen van een donor krijgt. Deze donor beenmergcellen vervangen het beenmerg van de patiënt, nadat deze uitgeschakeld is door chemotherapie. Een allogene stamcel transplantatie is een zware behandeling, waarbij patiënten soms zelfs kunnen overlijden aan de gevolgen van de therapie in plaats van aan de ziekte. Oudere patiënten, of patiënten met andere onderliggende ziektes (zoals long- of hartproblemen), komen daarom niet in aanmerking voor deze behandeling. Deze patiënten worden in studieverband behandeld met verschillende vormen van chemotherapie, of krijgen alleen ondersteunende therapieën zoals bloedtransfusies en antibiotica bij infecties.
In de inleiding van het tweede deel van dit proefschrift (hoofdstuk 6) wordt het schadelijke effect van bloedtransfusies beschreven. Transfusieafhankelijkheid kan worden gezien als een maat voor agressiviteit van de ziekte, maar mogelijk ook als maat voor toxiciteit van de transfusies zelf. Iedere eenheid bloed bevat ongeveer 200 mg ijzer - 100 keer de normale dagelijks benodigde hoeveelheid.

Omdat het lichaam geen fysiologische mechanismen heeft om dit overtollige ijzer te verwijderen, ontstaat als gevolg van veelvuldige transfusies ijzerstapeling (secundaire hemochromatose). Op korte termijn leidt dit tot orgaanschade en op lange termijn zou dit invloed kunnen hebben op de (leukemievrije) overleving. We hebben in dit proefschrift met name gekeken naar het overlevingsnadeel van transfusieafhankelijke patiënten die een allogene stamceltransplantatie ondergaan. Zo kijken we in hoofdstuk 8 en 9 naar factoren die de uitkomst van een stamceltransplantatie kunnen voorspellen. Dit zijn bijvoorbeeld de genoemde diagnostische WHO-classificaties, bepaalde chromosomale afwijkingen, het al hebben van andere ziektes (co-morbiditeit), maar dus ook het ondergaan van bloedtransfusies. We laten zien dat het toepassen van ijzer-reductie-maatregelen (met medicatie dan wel met aderlatingen) na de transplantatie een overlevingsvoordeel geven. Dit zal echter nog bevestigd moeten worden in een gerandomiseerde studie.

Conclusie
MDS is een complex ziektebeeld waarbij het stellen van de diagnose een continue uitdaging blijft. Dit proefschrift draagt op verschillende vlakken bij aan het oplossen van het diagnostische probleem. Het eerste deel van dit proefschrift dient als basis voor verdere verbetering van MDS-FC modellen. Modellen die zowel sensitief als specifiek zijn en bovenal kort en makkelijk te implementeren. Verder kunnen de artikelen dienen als hulpmiddel om MDS-FC te gaan gebruiken in andere laboratoria. We bieden in dit proefschrift handvatten om betere diagnostische categorieën te definiëren. Deze nieuwe classificatie zou dan weer kunnen dienen om de therapie beter af te stemmen en de keuze voor een bepaald geneesmiddel makkelijker te maken.
Het tweede deel van het proefschrift onderstreept de negatieve invloed van bloedtransfusies op de overleving na allogene stamceltransplantatie. De beste manier om tranfusieschade te verminderen is het verkorten van de periode tussen diagnose en curatieve (genezende) therapie. Betere prognostische modellen kunnen hierbij helpen doordat de therapie dan beter kan worden afgestemd op het te verwachten beloop van de ziekte.

APPENDIXES

CURRICULUM VITAE
LIST OF PUBLICATIONS
ACKNOWLEDGMENTS

Curriculum Vitae

Eline Marie Paulette Cremers werd geboren op 18 december 1985 te Roermond, Nederland. In 2004 behaalde zij haar Gymnasium diploma aan het Bisschoppelijk College Schöndeln, te Roermond. Hierna begon zij aan de opleiding Geneeskunde aan het VU Universitair Medisch Centrum (VUmc) in Amsterdam. Naast haar opleiding was zij betrokken bij onderzoek op het gebied van de Kinderoncologie. Ze deed onderzoek naar de gevolgen van ondervoeding bij kinderen met acute leukemie (prof. dr. Veerman; Manado, Indonesië) en onderzoek naar de lange termijneffecten van chemotherapie in kinderen (prof. dr. Gemke; VUmc). Gedurende haar co-schappen volgde ze extra co-schappen op de afdeling Hematologie, Radiotherapie en Neonatologie. In 2011 studeerde ze af, met als afstudeerprofiel Oncologie.
Na haar afstuderen begon ze als promovendus verbonden aan de afdeling Hematologie in het Radboud Universitair Medisch Centrum, te Nijmegen. Waar zij onder supervisie van prof. dr. T. de Witte begon met het alhier gepresenteerde onderzoek. Prof. dr. J.H. Jansen sloot aan in 2012.
In 2012 keerde Eline terug naar Amsterdam om onder supervisie van prof. dr. A.A. van de Loosdrecht en dr. ing. T. M. Westers het alhier gepresenteerd onderzoek voort te zetten.
Sinds september 2015 is zij in opleiding tot Internist in het Maastricht Universitair Medisch Centrum, te Maastricht (opleider prof. dr. Stehouwer). Momenteel is zij werkzaam in het Zuyderland Medisch Centrum te Heerlen (opleider dr. J. Buijs).

Eline Marie Paulette Cremers was born on December 18th 1985 in Roermond, the Netherlands. In 2004 she completed her secondary education (Gymnasium) at the Bisschoppelijk College Schöndeln, in Roermond. After that, she studied Medicine at the Vrije Universiteit in Amsterdam, the Netherlands. Besides her medical education she was involved in Pediatric Oncology research regarding influence of nutritional status in acute childhood leukemia (prof. dr. Veerman; Manado, Indonesia) and research regarding long-term effects of chemotherapy in children (prof. dr. Gemke; VUmc, Amsterdam, The Netherlands). During her internships she completed extra internships at the Department of Hematology, Radiotherapy and Neonatology, all at the VUmc. In 2011 she received her medical degree with the graduation profile: Oncology.
After graduating, she started her PhD research project that resulted in the current thesis, under supervision of prof. dr. T. de Witte at the Department of Hematology at the Radboud University Medical Centre in Nijmegen, the Netherlands. Prof. dr. J.H. Jansen joined the project in 2012.
In 2012, Eline returned to Amsterdam, were she continued to work under the

supervision of prof. dr. A.A. van de Loosdrecht and dr. ing. T. M. Westers, on the thesis presented here.
Since September 2015 she started her residency training in Internal Medicine at the Maastricht University Medical Center, Maastricht, the Netherlands. (head of the training committee: prof. dr. C. Stehouwer). She currently works at the Zuyderland Medical Center in Heerlen (head of training: dr. J. Buijs).

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